STANDARD OPERATING PROCEDURES

BACTERIOLOGY

VETERINARY SECTOR

Antimicrobial Resistance Surveillance and Research Network

Indian Council of Medical Research
Ansari Nagar, New Delhi-110029, India
2019

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Preface

'One Health is the collaborative effort of multiple health science professions, together with their related disciplines and institutions – working locally, nationally, and globally – to attain optimal health for people, domestic animals, wildlife, plants, and our environment.' ***One Health Commission***

Antimicrobial resistance (AMR) is now recognized globally as a public health challenge impacting all 'One Health' sectors i.e. humans, animals and environment. AMR is linked with high disease burden and economic consequences on people and nations. Addressing this challenge requires prioritized action and coordination across all government sectors, multiple stakeholders and society. It is well documented that high use of antimicrobials in humans and animals are contributing to increasing burden of drug resistant infections. Continuous surveillance of antimicrobial resistance in bacteria that cause infections in humans and animals are essential when studying changes in the antimicrobial susceptibility patterns of these organisms over time and to identify emerging resistance properties.

While the trends in AMR in humans and animals are well documented in developed countries, the data from developing countries is still lacking. This is due to many factors, key among them is lack of evidence due to absence of platforms for integrated surveillance. While the antimicrobial susceptibility testing in humans is well standardized, similar standardized guidelines are absent from veterinary sector. Also, there is no uniform harmonised methodology of antimicrobial susceptibility testing (AST) among the various surveillance and monitoring programs in human and veterinary medicine, globally.

Recognizing the lack of guidance to guide microbiologists and researchers on integrated surveillance of AMR in animals and humans, Indian council of medical research (ICMR) and Indian council of agricultural research (ICAR) with support from Food and Agricultural Organization (FAO), UN have created this Standard Operating Procedures (SOP) for veterinary microbiology labs. The SOP describes the regularly performed procedures of antimicrobial testing in bacteriology laboratory on clinically relevant gram-positive and gram-negative bacteria, methods of specimen collection, transport, culture, antimicrobial susceptibility testing (common, special phenotypic and molecular techniques) as well as quality control in a concise manner to ensure the quality assurance and consistency in the operation. Reference to any commercial method or equipment does not mean endorsement of ICMR; this is only for the purpose of this research study. The SOP aims to build the capacity for integrated surveillance of AMR in pathogen/commensals in food producing animals, food of animal origin and foodborne pathogens in humans and describes in detail. There is a complete chapter on breakpoints for both clinical specimen and surveillance specimen which has been adapted from CLSI, 2018 and customized as per Indian data.

We hope that this SOP will be adopted by various veterinary institutions in India to ensure uniformity in surveillance and epidemiological studies for AMR, resulting in reduction in the errors in data collection and thus allowing for meaningful analysis.

Dr. Kamini Walia,
Indian Council of Medical Research, New Delhi

Dr. B. R. Shome,
NIVEDI (ICAR), Bengaluru

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ABBREVIATIONS

AMA	Antimicrobial agent
AMR	Antimicrobial resistance
AMST/AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BGA	Brilliant green agar
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BP	Breakpoint
BPA	Baird Parker agar
BPW	Buffered peptone water
BSC	Biosafety cabinet
BSL	Biosafety level
CAZ	Ceftazidime
CD	Combined disk method
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CoNS	Coagulase negative staphylococci
CRE	Carbapenem resistant Enterobacteriaceae
DD	Double disk
DDST	Double-disk synergy test
DS	Double strength
DW	Distilled Water
eEDS	Extended EDS
EDS	EDTA disk synergy
EDTA	Ethylene diamine tetra acetic acid
ESBL	Extended spectrum β-lactamases
GES	Guiana extended spectrum β-lactamases
HCl	Hydrochloride
HLAR	High level aminoglycoside resistance
H_2S	Hydrogen sulphide
IPM	Imipenem
KPC	*Klebsiella pneumoniae* carbapenemase
LAI	Laboratory-acquired infections
LBM	Luria Bertani medium
MBC	Minimum bactericidal concentration

MBL	Metallo beta-lactamases
mCIM	Modified carbapenem inactivation method
MEM	Meropenem
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MPA	Mercaptopropionic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
NA	Nutrient agar
NaCl	Sodium chloride
NB	Nutrient broth
NDM	New Delhi metallo-β-lactamases
NS	Non-susceptible
NTS	Non-typhoidal *Salmonella*
ONPG	Ortho-nitrophenyl-β-galactopyranoside
ORSA	Oxacillin resistant *Staphylococcus aureus*
PBG	Phosphate buffered glycerin
PBP	Penicillin- binding protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Phenylalanine deaminase
PMQR	Plasmid mediated quinolone resistance
PPE	Personal protective equipment
PPS	Probability proportional-to-size
PSU	Primary sampling units
PYR	L-Ppyrollidonyl β-naphthylamide
QC	Quality control
RVS	Rappaport-Vassiliadis soy peptone broth
RT	Respiratory tract
SBA	Slanetz and bartley agar
SC	Selenite cysteine broth
Spp.	Species
SPS	Sodium polyethanol sulphonate
SRS	Simple random sampling
SST	Skin and soft tissue
SSU	Secondary sampling units
TSB	Tryptic soy broth

TSI	Triple sugar iron
UT	Urinary tract
VET	Veterinary
VIM	Verona integron-mediated metallo-β-lactamase
VP	Voges-Proskauer
XLD	Xylose lysine deoxycholate agar

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10	College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University (SDAU), S.K.Nagar, Gujarat
11	School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab

Amendment Sheet						
Sr. No.	**Page No.**	**Date of amendment**	**Amendment made**	**Reason**	**Signature**	**Signature lab Director**

General Guidelines

CHAPTER 1

GENERAL GUIDELINES

1.1 Scope

This is a comprehensive standard operative procedure manual for all types of specimens received in a veterinary bacteriology diagnostic laboratory of a veterinary institute or college. The manual is compiled by referring to international standards and protocols that are customized according to the needs and infrastructure, already available or achievable by up-gradation, in India. Both conventional and automated procedural alternatives are included. It is intended that all participating laboratories will strictly adhere to the procedures. The manual is structured to place each part of the procedure collectively in a stepwise manner comprising specimen collection, processing, antimicrobial susceptibility testing and quality control (QC) etc. Guidelines for specimen collection and transport should be provided to the collection points, and those for processing should be available in the processing laboratories. All the laboratories must isolate, identify pathogen to species level and perform susceptibility tests of significant bacterial isolates as per the provided guidelines.

1.2 Role of the laboratory

Microbiologists play a critical role in gathering of data for clinical and veterinary health decision making. Efficient and accurate microbiological diagnosis of bacterial infections guides the appropriate choice of antibiotics and other treatment alternatives. Similarly, microbiological surveillance is critical to guide the suitable antibiotic therapy through the identification of local resistance profiles. Thus, the role of microbiology laboratory is essential to manage various aspects of the animal health and prevent and treat bacterial infections.

1.3 Identification of occupational hazards

Several hazards *viz.* physical, biological or chemical can be encountered while working with animals. The primary hazards associated with the handling of animals include physical injury, animal allergens, zoonoses, respiratory hazard, eye hazard, sharp object related injuries, and dermatological conditions etc.

1.3.1 Bites, scratches, kicks

Bites, scratches and kicks are ever-present hazards associated with animal contact. Proper training in animal handling, general restraint techniques, familiarity with species-specific environmental factors and first aid procedures, and incident reporting are necessary to avoid such hazards.

1.3.2 Animal allergens

Animals or animal products such as dander, hair, scales, fur, saliva, and body wastes contain various allergens and exposure to them can cause respiratory (e.g. asthma), and skin disorders (e.g. allergies).

1.3.3 Zoonoses

Any disease that can be transmitted between animals and humans is termed a 'zoonotic' disease[1]. People can be exposed to zoonoses (bacteria, viruses, parasites or other infectious agents) in farms, veterinary hospitals, fields, as well as from the pets. These diseases pose a safety hazard to animal handlers if they are unaware and not trained about the prevention of disease transmission.

1.3.4 Sharp object related injuries

Personnel may be at risk of sharp-related injuries from needles, syringes, broken glass, and scalpels.

1.3.5 Eye Hazards

Eye safety concerns include direct contact with dust, other airborne contaminants or splash of infectious material (body fluids, urine etc.).

1.3.6 Respiratory hazards

Dust or an allergen from feed, bedding, manure, and many other sources when inhaled can cause severe respiratory problems.

1.3.7 Dermatologic conditions

Exposure to substances on animal's fur may result in a variety of dermatologic rashes, lesions, and other conditions.

The risks related to these hazards can be minimized through proper hazard identification, training, safe work practices, implementation of appropriate administrative controls, and judicious use of accurate protective environment. Exposure to hazard through a bite, scratch, needle stick, aerosol droplet, mucosal secretion, faeces or urine, requires immediate medical attention.

1.4 Biosafety

Laboratory personnel working with infectious agents are at risk of laboratory-acquired infections (LAI) due to accidental exposure or unrecognized incidents. The degree of hazard depends upon the virulence, dose of the biological agent, duration and route of exposure, host resistance, proper biosafety training, and experience in handling biohazards. LAI occur when microorganisms are inadvertently ingested (faecal/oral), inhaled (respiratory route), exposed to mucous membrane (eyes, nose, mouth), or introduced into

the tissues or skin or skin breaks (parenteral route) via cuts, bites, scratches, needle sticks, etc. Infectious agents may be present in body fluids, secretions such as blood, saliva, urine, faeces, respiratory secretions, and in animal tissues. Though LAI are not extensively reported, infections with pathogens are possible if appropriate biosafety procedures are not strictly followed in a well-equipped laboratory. Biosafety level 2 (BSL-2) practices are required for work involving these pathogens as they entail potential hazard to the personnel and the environment.

The following requirements are established for personnel working in the BSL-2 facilities[2]:

- Laboratory personnel must receive specific training in handling pathogenic agents, and should be supervised by trained and experienced scientists.
- Access to the laboratory must be limited to authorized personnel when work is being conducted.
- Personal protective equipment (PPE) must be worn at all times, and care must be taken to perform procedures that can create aerosols or splashes. These procedures should be conducted in biosafety cabinets (BSCs) or other physical containment equipment.
- Contaminated sharp items must be handled with extra precautions, and sharps should be disposed of in appropriately labeled hard plastic containers.

1.4.1 Protective clothing and equipment

a) Laboratory coats

Protective coats, gowns, smocks, coveralls or uniforms designated for laboratory use must be worn while working in the laboratory. Laboratory coats should fit properly and should cover arms to the wrist. The protective clothing must be removed and left in the laboratory before leaving for non-laboratory areas, such as offices or eating areas. Personnel should never take protective clothing to home, and it should be either disposed of in the laboratory or laundered by the institution.

b) Gloves

Gloves should be worn regardless of the type of infectious material while performing procedures involving potentially hazardous infectious materials that pose the risk of splashing or skin contamination, or contact with cuts or broken skin on hands of the laboratory worker. Gloves should always be worn for handling clinical specimens, body fluids, and tissues from animals. Arm length bite resistant gloves (heavy reinforced gloves of leather or similar material) or puncture resistant gloves (e.g. Kevlar and stainless-steel mesh) should be preferred. The sleeves of these gloves should extend up to or over the elbows offering protection of the hands and forearms. Moisture impermeable (vinyl, latex or nitrile) gloves should be used to decrease the contamination of skin by wet or dirty surfaces. Puncture resistant gloves can be worn

either over impermeable gloves or under other protective gloves to protect from bite or injury.

Gloves must be removed after completion of work with infectious materials or upon contamination by splashing or spills, or if integrity of gloves gets compromised. When removing gloves, avoid touching any area of the gloves that might be in contact with infectious material. Gloves should not be worn outside the laboratory. Personnel should not use telephone, mobile, computer, or open the doors with gloves that are used in laboratory procedures. Disposable gloves should never be washed or reused. All used gloves should be disposed of by autoclaving, and discarded along with other disposable materials. Hands should be washed immediately following removal of gloves.

c) Barrier precautions

Clinical specimens, body fluids, and tissues from animals should be assumed probably positive for pathogens. These materials should be handled in a BSC or using other barrier precautions (e.g. goggles, face mask, face shield, or other splatter guards) when a procedure can potentially create aerosols. Closed-toe comfortable shoes with low heels should be worn in the laboratory or other areas where chemicals are kept to reduce the possibility of injuries that may happen from spills, splashes, falling objects, slipping, and broken glass. Hearing protection equipment (e.g. foam plugs, ear muffs) should be worn to protect the ear when exposed to >85 decibels. A full face shield along with a fitted N95 face mask or other approved respirator should be used to protect the mouth, nose, eyes, upper-airways, bronchial and lungs from splashes, droplets and aerosols when necessary. Stretch booties usually made of paper or plastic should be worn over street shoes to protect from contamination, and these should not be worn outside the animal facility. A dedicated facility for footwear may be substituted for shoe coverings, if resources are available.

1.4.2 Standard microbiological safety practices

The following safety guidelines apply to all microbiology laboratories regardless of biosafety level. All procedures involving biological materials, potentially infectious materials, or clinical specimens should be performed while wearing appropriate PPE.

a. Restrictive access to laboratory

Access to the laboratory should be limited to trained personnel during the work regardless of the settings. The access to un-authorized personnel should be restricted, and a separate section should be maintained to interact with non-laboratory personnel. Biohazard signs or stickers should be pasted on all laboratory doors, and equipments used for laboratory work (e.g. incubator, BSC, microwave, ice machine, refrigerator, and freezer etc.). Minor children and pets should not be allowed in laboratory areas. All

laboratories should be locked when not in use. In addition, all freezers and refrigerators located outside in corridors should be locked, especially those that contain infectious pathogens or other hazardous materials.

b. Autoclaving

An autoclave must be available in the BSL-2 laboratory and should be operated by trained personnel only. To verify the proper working of autoclave, spore strips (e.g. *Geobacillus stearothermophilus*) or other biological indicators designed to test the efficiency of sterilization should be included in autoclave loads on a regular basis (i.e., monthly). Each batch for autoclaving should be monitored with temperature-sensitive tape or thermograph or by other means (i.e. biological indicators). A logbook should be maintained to record the date, time, and indicator of sterilization for each batch.

c. Disinfection

Organisms may harbor different susceptibilities to various disinfectants. As a surface disinfectant, 70% isopropyl alcohol is generally effective; however, it is not preferred for the decontamination of spills. It should be noted that 100% alcohol is not as effective a disinfectant as 70% alcohol. Phenolic disinfectants, although expensive, are effective against many organisms. Always read disinfectant labels carefully for manufacturer's recommendations for dilution and exposure times for efficacy. An effective general disinfectant is a 1:100 dilution (1%) of household bleach (sodium hypochlorite) in water. At this dilution, bleach can be used for wiping surfaces of benches, hoods, and other equipments. The diluted solutions of bleach should be prepared weekly from a concentrated stock solution. A 1:10 dilution (10%) of bleach should be used to clean up the spills of culture or concentrated infectious material or any serious contamination. However, 10% bleach can pit stainless steel owing to its corrosive nature; hence it should not be used routinely whereas, 70% alcohol should be used to deactivate the bleach.

d. Disposal of contaminated materials

All discarded petri plates; tubes, clinical samples, pipettes, gloves, and other contaminated materials should be placed in disposal containers. Special disposal containers, typically constructed of puncture-proof plastic, must be used for sharps to minimize the risk of injury. Avoid overfilling of disposal containers. The lids should rest with flush top containers. The containers of contaminated material should be carefully transported to the autoclave room and autoclaved before disposal. Water should be added to each container to be autoclaved, for optimal sterilization. Waste disposal containers in the laboratory should be clearly labeled for disposal of infectious items or non-infectious items. Waste disposal containers for infectious or potentially infectious items should be lined with a plastic biohazard or otherwise specially marked bag.

e. Decontaminating bench tops and other surfaces

Bench tops and other potentially contaminated surfaces should be wiped with a disinfectant (10% bleach) routinely after working with infectious agents or clinical specimens. In case of spill, splash or contamination by infectious materials, affected area must be decontaminated properly with disinfectant. Following disinfection with 10% bleach, the surface must be wiped down with 70% isopropyl or ethyl alcohol to inactivate the bleach and prevent corrosion of the work surface. The solutions of disinfectants should be maintained at each work station.

f. General laboratory cleanliness

All areas of the laboratory should be organized and must be clean. Safety hazards such as dirt, dust, crowding, clutter, considered unacceptable for biological research and may lead to contamination of specimens, isolates, and biological assays. Floors should be regularly dry mopped followed by wet mop using germicidal solution.

g. Decontamination of spills

Management of biological spills must account for the specific infectious agent (if known), volume of spilled material, and the presence of aerosols. The following procedure is recommended for decontamination of the spills.

i. Isolate the area to prevent anyone from entering.
ii. Wear gloves and protective clothing such as a gown or lab coat, shoes, and a mask (spill may contain a respiratory agent or agent is unknown).
iii. Absorb or cover the spill with disposable towels, but do not wipe up the spill or remove the towels.
iv. Saturate the disposable towels and the affected area with an appropriately (intermediate or high level) diluted disinfectant (e.g. a phenolic formulation or household 10% bleach), and leave them in place for at least 15 minutes.
v. Wipe area using clean disinfectant-soaked towels, and allow to air dry.
vi. Place all disposable materials used to decontaminate the spill into a biohazard container. Use mechanical means to dispose of broken glassware.
vii. Handle the waste material in the same manner as other infectious waste.

If a spill occurs in BSC, do not turn off the cabinet fan. Use absorbent paper to soak minor spills. If spill flows into the grille, wipe all the items with disinfectant and remove them. Close the drain valve and pour disinfectant onto the surface and through the grille into the drain pan. Allow appropriate contact time, then drain, rinse and dry. If breakage occurs in a centrifuge, keep the centrifuge tightly closed for 30 minutes to contain aerosols before decontamination.

h. Hand washing

All laboratories should contain a sink with running water and soap for hand washing. Frequent hand washing is one of the most effective procedures to reduce the dissemination of the infectious agent for avoiding LAI. Hands should be washed for at least one minute with an appropriate germicidal soap after handling infectious materials

(viz., infected animals, or coming in contact with animal saliva, urine, faeces, and blood) before exiting the laboratory. If germicidal soap is unavailable, then use 70% isopropyl or ethyl alcohol to clean hands.

i. Mouth pipetting

Mouth pipetting is strictly prohibited. Rubber bulbs or mechanical devices must be used.

j. Sharps

Thorough precautions must always be taken with any contaminated sharp items including needles, syringes, slides, glass pipettes, capillary tubes, broken glassware and scalpels. Sharps should be disposed of in designated puncture-proof, leak-proof and sealable sharps containers. To minimize finger sticks, used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal. Non-disposable sharps should be placed in a labeled discard pan for decontamination before cleaning. Broken glassware should be removed by mechanical means (e.g. brush and dustpan, tongs, or forceps), and should not be handled directly by bare or gloved hand.

k. Aerosols

All procedures must be carefully performed to minimize splashes or aerosolization. Laboratory work should be conducted in a BSC or by wearing the appropriate PPE (e.g. goggles, mask, face shield, or other splatter guards). The procedures involving potentially infectious aerosols formation, splashing or spraying of the face with infectious or other hazardous materials, face protection mask should be used while working with high concentrations or large volumes of infectious agents.

Procedures that pose such risks may include:

i. Centrifugation, vortex, and vigorous mixing: these procedures should be performed in closed containers. Sealed tubes should be used if safety-capped tubes are not available.
ii. All body fluids and infectious materials should only be centrifuged in carriers with safety caps.
iii. Handling tissue specimens or body fluids: gauze should be used to remove the tops on blood specimens and should be placed around the top of blood culture bottles to minimize aerosol production during removal of the needle. Grinding of tissue specimens should be performed in a BSC.
iv. Sonic disruption: infectious materials that undergo sonic disruption should be placed in a sealed container within the sonicator.
v. Opening containers of infectious materials whose internal pressures or temperatures may be different from ambient pressures or temperatures.
vi. Loops containing infectious material should be dried in the hot air above a burner before flaming.

vii. Inoculating wires and loops should be cooled after flame sterilization by holding them still in the air for 5-10 seconds before they touch colonies or clinical material. Disposable loops should be preferred if resources are available.

l. Refrigerators and freezers

The temperature of laboratory refrigerators and freezers should be monitored daily to ensure proper functioning. Regular inspections should be done for the presence of broken vials or tubes containing infectious agents. When removing and discarding broken material, laboratorians should always wear gloves and PPE. Disinfectant should be applied at the affected area, and kept in place for at least 15 minutes before removal of the broken infectious material. Refrigerators and freezers should be regularly cleaned with a disinfectant and defrosted to prevent possible contamination or temperature failure.

m. Fire prevention

Burners should be used away from light fixtures and flammable materials. Bulk flammable material must be stored in a safety cabinet. Small amounts of these flammable materials (e.g. ethyl acetate, ethyl alcohol, and methanol) can be stored in safety containers such as a safety bench canister or dispenser. Burners must be turned off when not in use. All personnel must know the location of fire extinguishers, fire blankets, alarms, and showers. Fire safety instructions and evacuation routes should be posted at relevant areas. However, usage of burner is prohibited in BSC-2 onwards.

n. Personal belongings

Eating, drinking, and smoking are not permitted in laboratory work areas. Food must be stored and eaten outside the laboratory in areas designated for that purpose only. Personal articles (e.g. handbags, eyeglasses or wallets) should not be placed on laboratory workstations.

1.4.3 Special practices

a. Care for animal bites and scratches

Handlers should watch the animal behavioral signals closely and must understand the animal signs of agitation. Appropriate safe facilities should be used and PPE must be worn to avoid bite and scratches. Animal handler should undergo the rabies pre-exposure vaccination doses as per the WHO guidelines before directly handling animals or dogs that may be infected with rabies. All bite wounds and scratches should be cleaned thoroughly with soap and water.

Bite injury should be reported to supervisor or professional expert. Medical evaluation of bite wound should be performed immediately to assess the possible exposure to pathogen and need for treatment or vaccination. All bites or scratches should be monitored for infection, and appropriate intervention must be provided if infection occurs, especially redness, inflammation, or swelling progresses rapidly.

b. Prevention of allergy hazard and zoonoses

Exposure to allergens can be avoided with the use of PPE such as gloves, gowns, hair/shoe covers, N95 respirator masks, and safety goggles etc. General laboratory cleanliness and safe work practices such as hand-washing or showering after handling animals or specimens (e.g. manure, urine, milk, meat, wool, rumen samples, blood, fluids from wounds etc.) should be implemented to prevent allergies and zoonoses. Personnel should never eat or drink anything during sample collection or in areas where animals, animal wastes, or animal products handled. Sickness in animals must be reported to veterinarian to determine the possible cause of the illness, and to adopt additional protective measures, if necessary. A designated pair of shoes and jeans or coveralls should be worn while working at the farm, and coveralls should be washed at the farm unit to reduce the risk of contamination.

c. Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. When cuts or puncture wounds from potentially infected needles or glassware occur, the affected area should be promptly washed with disinfectant soap and water for 15 minutes. Immediately report a needle-stick injury or any other skin puncture to the supervisor and appropriate health officials as prophylactic treatment of the personnel performing the procedure may be indicated. In the event of a centrifuge accident in which safety carriers were not used, other personnel in the area should be warned immediately and the area should be isolated to prevent anyone from entering.

d. Laboratory design and equipment

The laboratory should be designed to avoid conditions that pose biosafety problems. Access into the microbiology section must be restricted to staff only. Space should be ample and adequately illuminated for the safe circulation of staff during work. The areas for infectious and non-infectious work should be clearly separated. The receiving and set-up areas should be designed to accommodate the greatest anticipated number of specimens. Walls, ceiling, floors, benches, and chairs should be easy to clean, impermeable to liquids, and resistant to chemicals and disinfectants. Hand-washing basins with running water, soap and disinfectant must be provided in each room. An autoclave or other decontamination facility should be available close to the laboratory. Adequate storage space for specimens, reagents, supplies, quality control testing, record storage or personal items should be provided inside and outside the working area. An adequate sit-down work spaces equipped with computers should also be provided to the staff. Safety systems for fire, chemicals, electrical or radiation emergencies, and an emergency shower and eyewash facilities should be in place.

Security measures should also prevent theft, misuse or deliberate release of the infectious materials.

e. Medical surveillance of laboratory workers

The employing authority is responsible for providing adequate surveillance and management of occupationally acquired infections. Pre-employment and periodic health checks should be organized and performed. Prophylaxis or other specific protective measures may be applied after health check of the individual(s) and risk assessment of the possible exposure. Special attention should be paid to women of childbearing age and pregnant women as some microorganisms present a higher risk for the fetus.

Immunization of laboratory workers can also be proposed based on the following criteria:

- Conclusion of the risk assessment
- Verification of the immunization status of the worker by serology (immunity from prior vaccination or infection)
- Local availability, licensing state and utility of vaccines (i.e. does it provide protection against the prevalent serogroups or serotypes circulating in the region?)
- Availability of therapeutic drugs (i.e. antibiotics) in case of accident
- Existence of national regulations or recommendations
- A first-aid box containing basic medical supplies should be available along with a written emergency procedure to access a doctor for definitive treatment of the injury. First aid kits should be periodically checked for the expiration date.

1.4.4 Laboratory safety instructions

- Do not eat or drink in the laboratory.
- Wear laboratory coats and gloves while working in the laboratory.
- Wipe the working area with a disinfectant at the beginning and end of the work.
- Always wash your hands before leaving the laboratory.
- Avoid any activity that introduces objects into the mouth, e.g. mouth pipetting.
- Cover exposed skin surfaces or open cuts on hands with a water-resistant dressing.
- Perform all procedures in a way so as to minimize the risks of spills, splashes and the production of aerosols.
- No infectious material should be discarded down laboratory sinks or any other drain.
- Benches should be clear of all non-essential materials including books and notes.
- Lab fumigation should be done at regular intervals using formaldehyde and $KMNO_4$.

- Any symptom(s) of infectious disease or zoonosis following sample collection should be reported and never overlooked. A consultation with a healthcare provider for any suspected occupational illness should be of utmost importance.

1.4.5 Emergency measures: mishaps (spillage or minor spills) with infective material

Put on gloves, cover the spill with a cloth or tissue soaked in disinfectant, leave for 10 minutes and then mop up. If there is a gross spillage or any spillage with a specimen likely to contain a category 3 organism outside the safety cabinet, evacuate the room for at least an hour to allow possible aerosols to be dispersed and then fumigate the room. For category 2 organisms, disinfect and clean up. Deal with spillages in BSC by disinfecting the affected surfaces. Fumigate the cabinet for spillage of category 3 organisms. Encourage cuts and punctured wounds to bleed, and then wash with soap and water. If eye splash happens, rinse immediately with tap water or irrigating solution from the laboratory first aid kit. If the skin soiled with infective material, rinse with 70% alcohol or dilute hypochlorite solution, and then wash with soap and water.

Specimen Collection, Transport and Processing

CHAPTER 2

SPECIMEN COLLECTION, TRANSPORT AND PROCESSING

2.1 Introduction

Appropriate sample collection is one of the prerequisite for the accurate diagnosis of an infectious pathogen or the disease suspected and for health surveillance. For antimicrobial resistance (AMR) surveillance, it is very essential to collect appropriate sample for processing in the laboratory and subsequent testing for antimicrobial drug sensitivity.

2.2 General information on sampling

2.2.1 Sample type

Various specimens/clinical samples should be collected according to the source and purpose of sampling. During AMR testing and surveillance, the type of sample may vary according to the nature of the sampling, *viz*., sampling from animals/chicken, sampling from food of animal origin (meat/egg).

- **Blood:** Blood samples are collected for certain blood-borne pathogens and processed in the laboratory for screening of infectious agents and AMR testing. During collection of the blood sample, appropriate sterility measures should be taken to avoid any surface contamination during venepuncture. Blood can be collected by sterile syringe and needle or with vacutainer tubes. Ideally, the skin at the site of venepuncture should be swabbed with 70% ethyl alcohol, allowed to dry, cleaned by shaving or plucking the feathers and then swabbed again and allowed to dry. Various anticoagulants like ethylene diamine tetra acetic acid (EDTA; purple tube) and heparin (green tube) as required can be used to store and transport the blood samples. However, blood for culture should be collected in blood culture bottles and anticoagulants should be added directly after collection. In most of the large mammals, the jugular vein and caudal vein (in cattle) is selected as the site of blood collection. Occasionally, brachial vein, brachiocephalic vein, femoral vein, tarsal vein, mammary vein is used besides the cranial vena cava and ear vein in pigs, saphenous veins, retro-orbital veins, intracardial puncture in rodents and wing vein (brachial vein) in birds.
- **Pus:** In certain infections caused by pyogenic organism, pus is the apt sample for collection and screening for the presence of pathogen. Pus samples should be

transported without any transport media. In case of pus swab, Amie's transport medium can be used unless processed within 2 hours.

- **Urine:** Urine samples are collected for the screening of AMR spread through pathogens affecting the urogenital tract. Such samples should be transported in leak-proof container without addition of any preservatives, preferably refrigertaed.
- **Faeces:** During collection of the faecal material, care should be taken to avoid any external environmental contamination. Faecal samples should be collected from freshly defecated faeces. This faecal material should be sent in the zip-lock cover or leak-proof container with or without a transport medium. Collection of the sample directly from the rectum or cloaca through sterile measures ensures a better sterility from any possible environmental contamination.
- **Skin tissue:** Sample like skin scrapings should be collected and sent in appropriate zip-lock cover or sample containers. These samples can be sent at ordinary room temperature. Sampling from skin can include the vesicular skin materials or rashes, whenever necessary.
- **Milk:** Spread of AMR through milk is a serious concern for the human health. Hence, collection of the milk samples especially in case of mastitis (clinical/subclinical) from animals is crucial. The milk sample should be collected after proper cleansing and drying of the tip of the teat. The initial stream of milk should be discarded to avoid the collection of any environmental pathogens from the teat canal. Samples can be collected either from the affected quarter or all four quarters. Samples from each quarter should be collected separately. No preservatives should be added if milk samples are transported within same day to the laboratory. Alternatively, milk samples should be transported at a freezing temperature to the laboratory situated at a distant place.
- **Food of animal origin (meat/egg):** Collection of the meat of various animal species/poultry is important to monitor the food borne pathogens or infection, and spread of AMR through food sources. Proper sterile condition should be maintained to avoid any environmental contamination during sampling of meat or egg from the road side vendors, butcher shops and slaughter houses.

2.2.2 Sample preservatives and transport media

Various preservatives are used depending on the type of specimen or conditions. After proper labeling and packaging, preserved specimen must be dispatched **(Annexure I)** to the laboratory**.** The common preservatives are phosphate buffered glycerin (PBG) for tissue specimens collected from organs; EDTA, sodium citrate, heparin etc. for whole blood samples (Blood samples should be inoculated to blood culture bottles at the site of collection). For automated systems, it should be transported at room temperature till put in the machine. Conventional bottles should be transported at room temperature or kept

at 35 degree incubator till further processing; transport media like Cary-Blair medium for faecal samples; peptone water for gram negative and mannitol salt broth for gram positive pathogens for swab samples. The composition of various transport media are listed in the **Appendix I**.

2.2.3 Information to be sent with specimen

Collection agent should update the details of the sample information during sampling without fail. Structured questionnaire **(Annexure II)** should be filled, reviewed and submitted to the laboratory along with all the specimens**.**
Note: For multiple samples, send the separate sampling -proforma whenever required.

2.3 Specimen's type

- **Samples from animals and poultry**

2.3.1 Animal samples

This section refers to the sampling procedures from both healthy and diseased animals (Fig. 2.1). Animals are grouped as **domestic animals** (cow, buffalo and pig) and **pet animal** (dog).

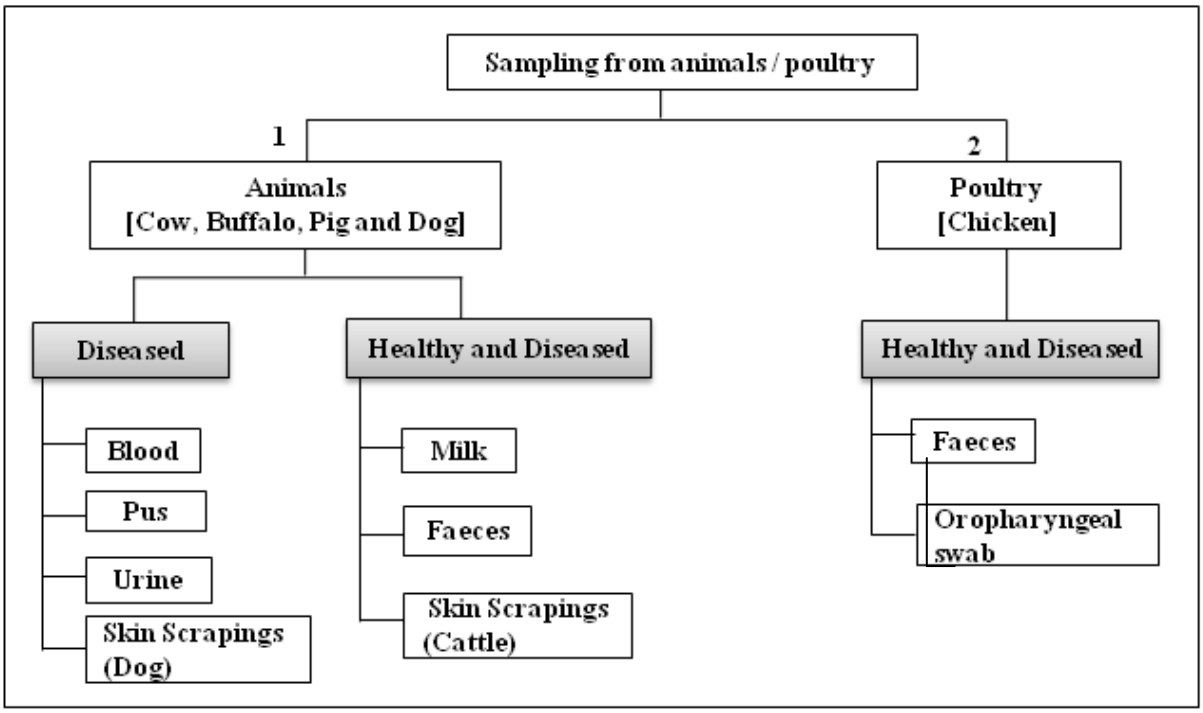

Fig 2.1: Flow chart illustrating specimen types from healthy and diseased animals

2.3.1.1 Skin scrapings[3]

Purpose: To isolate the potentially pathogenic organism to aid the diagnosis of certain skin inflammations and infections**.** This may also help in revealing the presence of abnormal cells in the superficial layers of the skin.

Specimen collection

a. Clean the site to remove superficial debris.
b. Collect the skin sample using a sterile scalpel blade.
c. Gently scrape layers of skin using blade and without causing any hemorrhage.
d. Keep skin scraping sample in screw capped bottle or test tubes or petri dishes by scrapping against inside the bottle or dish.

Transport

a. For smaller distance and same day processing, samples should be collected in a sterile container without any transport media over ice.
b. For longer distance, samples must be collected in transport medium or BPW, carefully packed and transported within 24 hours of collection under ambient temperature.
c. Screw capped bottles should be additionally sealed with adhesive tape or paraffin box.

Processing

a. Inoculate the skin scraping sample in BPW (1/10 dilution) for pre-enrichment.
b. Isolation of specific pathogens should be performed as mentioned in section 2.4.

2.3.1.2 Blood

Purpose: Blood samples should be collected for the isolation of blood-borne pathogens. These samples are further processed in the laboratory for screening of infectious agents and antimicrobial susceptibility testing (AMST)[4].

Prepare the blood culture bottle

Remove the septum of the blood culture bottle and the rubber stoppers on bottles or tubes. Appropriately label the bottles.

I Collection of blood from cattle

a. Wear gloves and select the venipuncture site.
b. In cattle of all ages, collect blood either from the jugular (neck) vein or from the coccygeal (tail) vein of older cattle.
c. Apply an antiseptic to clean the skin surface and allow drying prior to venipuncture.

- **Jugular bleeding**

i. Restrain cow using the halter to elevate head and expose the jugular groove.

ii. Put pressure in the jugular groove and raise the jugular vein.
iii. Insert the needle into the exposed vein and collect blood sample (~5 ml).
iv. Remove the pressure after collecting sufficient volume and apply gentle compression for a few seconds to stop the bleeding. Place bandage over the puncture.

- **Coccygeal bleeding**

i. Restrain the cow and raise the tail vertically to access the ventral surface.
ii. Clean the ventral surface of the tail using swab dipped in 70% ethyl alcohol.
iii. Select the site approximately 150 mm from the base of the tail.
iv. Insert needle perpendicular to the skin surface on the midline, approximately, between third and fourth coccygeal vertebrae.
v. Collect sufficient blood sample in vacutainer tubes and withdraw the needle.
vi. Apply gentle pressure to the venipuncture site and release the tail.

II. Collection of blood from pig

a. Restrain the pig manually. For restrainer, snout snare is recommended for pig over 20 kg and handling by experienced personnel only.
b. Clean the venipuncture site with alcohol swab, and allow drying.
c. Apply gentle pressure to the selected vein and collect blood sample (~5-10 ml) from the external jugular vein using sterile needle.
d. Remove the pressure from vein and press gently to stop bleeding.
e. An alternative venipuncture site is the caudal auricular ('marginal ear') vein that is easily visible on pig of any size. However, low volume (~1 ml or less) of blood can be collected from this site. Use a smaller (22G or 23G) needle for this vein.

III. Collection of blood from dog

a. Restrain the dog manually.
b. Blood can be collected either from cephalic vein or jugular vein.
c. Needle size should be selected according to the size of dog, and type of vein for venipuncture.
d. Shave the collection site and clean the area with alcohol swab or by spraying alcohol.
e. When withdrawing a sample from the jugular vein, shaving is usually not required, unless dog has excessive hair.
f. Collect the blood sample (2-5 ml) from jugular vein using sterile needle. For high sample volume, sampling sites can be alternated between the two jugular veins starting at base of the neck and moving towards the head.
g. For collection of blood from the cephalic vein, insert a wider needle over the raised vein of forelimb and pull back the syringe plunger to take out 2-5 ml of blood sample at once.
h. Remove the needle by release of pressure over the vein.

i. Gently press the venipuncture site for 30 seconds, and apply a disinfectant.

Transport of blood samples

a. In case of delay between collection and processing of sample, the bottles/samples should be kept at 35ºC incubator, if available. Otherwise, leave the bottle at room temperature. For automated culture system, the inoculated bottle should be kept at room temp till in the machine.

b. The bottles with blood samples should never be refrigerated.

Processing[2]

a. Safety

i. Keep the culture bottles within a biosafety cabinet or behind a shield, or wear a facemask. Always wear gloves.

ii. Use needleless transfer devices or safety needles, and never recap them.

iii. Dispose of needles and syringes in a puncture-proof container.

b. Incubate blood cultures for the predetermined period at 35 °C (usually 5 days, unless quality monitors indicate less time).

c. Maintain incubation conditions to allow recovery of microorganisms (as per manufacturer's instructions in case of automated system) and maintain adequate rotation or agitation of the media, when required.

d. Examine the cultures daily, whether detection of positives is by visual inspection or by an automated system. For visual inspection, observe for hemolysis, turbidity, gas production, puffballs, and clotting which are indicative of microbial growth.

Table 2.1: Examination of microbial growth

Microscopic observation	Associated microorganism
Hemolysis	*Staphylococcus*
Turbidity	Staphylococci, certain aerobic gram-negative bacilli
Gas formation	certain aerobic gram-negative bacilli
Clotting	*Staphylococcus*
Visible colonies (Puffballs formation)	*Staphylococcus*

e. For conventional method, blood culture needs to be carried out in two bottles containing 50 ml each of tryptone soya broth and bile broth. After removing the kraft paper, inoculate culture bottles with blood sample. Incubate at 37 °C and examine daily for 7 days for the evidence of growth that is indicated by turbidity, hemolysis, gas production, discrete colonies, or a combination of these.

f. For manual broth systems, perform at least one blind subculture to appropriate solid agar from visually negative bottles. Perform blind subculture after 1st overnight

incubation and on 3rd and 7th day post-incubation on sheep blood agar and McConkey agar. NOTE: Subculture of automated systems has little clinical utility.

g. In special circumstances, when culture appears to be negative, perform a gram stain, wet mount, or acridine orange stain from the culture or its sediment to determine the presence of microorganisms.

h. Discard positive and negative bottles safely after autoclaving at 121 ºC for 60 minutes. After autoclaving, open the automated system bottles with an opener and discard the inoculated medium in a designated shank for biomedical waste. For manual systems, open the screw capped bottles and similarly discards the inoculated medium**.**

Further processing of the positive blood cultures

a. Gram-stain a thin smear from the broth or agar immediately when suggestive of growth.

b. Subculture to agar media and put up biochemical tests based on the Gram stain results.

Limitations of blood culture

1. Low levels of organisms may not be detected in the incubation interval of the culture.

2. Used media may not support the growth of some organisms. Use of multiple formulations increases the yield.

3. Sodium polyethanol sulphonate (SPS) may inhibit the growth and viability of the organism.

4. Bacterial metabolism may not produce sufficient CO_2 for detection in automated systems.

5. There are a number of fastidious microorganisms that infect the blood, which cannot be grown in routine blood culture.

2.3.1.3 Pus

Purpose: To isolate and identify bacterial etiological agent(s) in deep seated pus specimen.

Specimen collection

a. Pus should be collected either by swab method or by aspirating exudates using a syringe and needle.

b. From a superficial wound, pus specimen should be collected using a sterile dry swab.

c. Insert a swab to rub/roll on infected tissue and keep swab sample in a sterile vial.

d. From a deeper wound, aspirate the exudates into a syringe and collect it in a sterile tube or vial.

Transport

a. Swab samples or exudates should be transported to the lab as soon as possible.

b. Samples should not be refrigerated or incubated before or during the transport.

c. Swab samples should be transported with sterile BPW at ambient temperature.

Processing

a. Samples should be diluted or pre-enriched with peptone water/ media according to gram positive or gram-negative pathogen isolation.
b. For pre-enrichment dilute swab suspension to 1/10 in BPW.
c. Isolation of specific pathogens should be performed as mentioned in section 2.4.

2.3.1.4 Urine

Purpose: To isolate the pathogenic organisms from urine samples aiding in the diagnosis of infections.

Collection of urine

Urine should be collected as free catch/voided sample; urethral catheterization and cystocentesis. Each urine specimen received by the laboratory should be clearly labeled[5].

- **Free catch/voided urine**

a. Midstream clean catch urine is the most common and easiest method of urine collection, however it can be challenging sometimes depending on the animal.
b. Midstream collection requires voiding the first portion of urine which is most likely to be contaminated by urethral commensals.
c. Urine sample should be collected in sterile wide mouthed screw capped bottle/container.

- **Urethral catheterization**

a. This technique is a common method of urine collection in dogs, and may require sedation of the animal.
b. Before urine collection, all materials should be sterilized and collector should wear gloves.
c. A urinary catheter is introduced in the distal end of urethra to drain urine from the bladder.
d. Samples are collected directly into a specimen container.

- **Cystocentesis**

a. This technique is most suitable to collect urine for laboratory testing mainly for culture.
b. In this technique animal can be positioned on its back, standing or propped up on its hind legs depending on the size and gender of the animal.

c. Prior to the sample collection, careful palpation of the urinary bladder should be done to check its size. After palpation, isolate the bladder with one hand and insert a fine gauge needle (at 45 °C) into the bladder via the abdominal wall.
d. Urine sample is collected into a sterile container/wide mouthed bottle.

Transport
a. Urine sample should be transported to the laboratory on ice as soon as possible.
b. Sample should be processed as early as possible after the collection.
c. In case of delay in processing, sample should be refrigerated up to maximum 24 hours.

Processing
Inoculate or streak urine samples on the suitable growth medium for isolation of pathogens (section 2.4).

2.3.1.5 Faeces

Purpose: To describe the collection and processing of faeces in the laboratory for microbiological examination, and to isolate the potentially pathogenic organisms aiding in the diagnosis of infections[3].

General considerations
a. Prior to faecal collection, ensure that the animals are properly restrained.
b. It is preferable to collect the fresh faecal samples from rectum.
c. Care should be taken to collect the faecal sample from the mid deep portion of the faeces voided, while avoiding the surface and peripheral part, in order to avoid any chance of environmental contamination
d. In case of sample collection from the ground, it should be from the top of a freshly passed deposit.
e. Minimum 5-10 g of faeces sample should be collected for the isolation of bacteria.

Faecal sample collection from the rectum of cattle
a. Operator should wear long gloves on both hands.
b. After restraint, hold the animal's tail to one side.
c. Gently insert a gloved hand through the anus by applying pressure to the anal sphincter.
d. After penetration into the rectum, massage the wall in a circular motion that induces the evacuation of faecal matter.
e. Collect faecal material in sufficient amount in a sterile container or zip lock bag.

Faecal sample collection from the rectum of calf, buffalo calf and piglet
a. Restrain the animal manually and gently inserts a gloved lubricated finger through the anus. If faeces are not produced, then use finger for collection.

b. In case of difficulty in collecting faeces sample from small animals, use sterile swabs. Insert the swab and gently swab the rectal wall by rolling several times.
c. Keep the swab samples in buffered peptone water (BPW) or Mannitol salt broth.

Transport of faecal sample

a. Samples should be transported to laboratory within 8 hours of collection. In case of delay, samples should be refrigerated or stored on dry ice.
b. Double packaging of samples is advisable in order to prevent spillage and cross contamination.
c. Faecal collection in plastic bag or rubber stopper tubes should be avoided as bacterial growth in samples may cause gas production leading to leakage of the specimen.

Processing of faecal sample

a. All the samples should be stored at 4^{o}C until processed for the isolation of bacteria.
b. Samples can be diluted or pre-enriched with peptone water/media depending upon gram positive or gram negative pathogen isolation.
c. For pre-enrichment, take a minimum of 10 g faeces sample for 1/10 dilution in BPW (if ample amount of sample is available, then 25 g of faeces sample in 225 ml of BPW should be preferred), and homogenize it.
d. For swab samples, dilute the swab suspension to 1/10 in BPW.
e. Isolation of specific pathogens should be performed as mentioned in section 2.4.

2.3.1.6 Milk

Purpose: To describe the collection and processing of milk samples from healthy and diseased animals for microbiological examination, and to isolate pathogenic organisms aiding in the diagnosis of mastitis and other infections[6].

Collection of milk samples

a. Milk samples should be collected aseptically before milking.
b. Milk samples from healthy cattle can be collected at random from one of the hind quarters.
c. When individual quarter shows clinical sign of mastitis, milk samples should be collected from all the affected quarters separately.
d. Discard the initial stream of milk and pre-dip the teat in 0.5% iodine or 4% hypochlorite for 20-30 seconds.
e. Clean and dry the teat carefully to make sure no pre-dip left behind on the teat, and then disinfect using 70% ethyl alcohol.
f. Discard 3-4 streams of milk and collect the sample (3-5 ml) directly into sterile vial or container without any preservative.

Transport

a. Label the sample and immediately keep on ice until transported to the laboratory.

b. Samples should be frozen, if not transported to the laboratory within 24 hours.

Processing

a. Keep samples at 4 °C until processed for bacteriological analysis.

b. Inoculate milk sample on selective growth medium using sterile cotton swabs for isolation of specific pathogens (section 2.4).

2.3.2 Poultry samples

This section refers to sampling procedures from both healthy and diseased chicken.

2.3.2.1 Faeces

Collection and Transport

a. Faecal specimen should be collected from the ground or using swab from the cloacal region of chicken.

b. In case of faecal samples collected from the ground, it should be from the top of a freshly passed deposit.

c. For cloacal swab method, insert a sterile swab into the vent and gently swab it by rolling several times against the mucosal wall.

d. Gently remove the swab after deeply stained with faecal material and place it in the sterile tubes containing 2-5 ml BPW or Cary-Blair medium.

e. Samples should be packed carefully and transported immediately to laboratory at 4^{o}C.

Processing

a. All samples must be preserved at 4^{o}C until processed for bacterial isolation.

b. Processing steps are similar to the processing of faecal sample for animals (section 2.3.1.5).

2.3.2.2 Oropharyngeal swab

Purpose: To describe the collection and processing of swab samples from the healthy and diseased animals for microbiological examination, and to isolate pathogenic organisms aiding in the diagnosis of infections.

General Considerations:

a. Always use separate swab for each bird.

b. Avoid touching other areas with the swab in order to prevent contamination.

c. A moistened (in sterile water) swab should be used for healthy birds to avoid excessive tissue damage.

Collection and Transport

a. The operator should try opening the beak with one hand, and inserting a sterile swab into the oropharynx region.

b. Swab the wall by rolling the swab gently several times.

c. Remove the swab by gentle rotation and place in the sterile tube containing BPW or mannitol salt broth.

d. Samples should be packaged and immediately transported to the laboratory at $4^{o}C$.

Processing

a. All the samples must be preserved at $4^{o}C$ until processed for bacterial isolation. Perform isolation of specific pathogens as mentioned in section 2.4.

2.3.3 Food samples of animal origin

Purpose: To undertake AST in food borne bacteria as food samples of animal origin (Fig 2.2) represent the major route of human exposure to food-borne pathogens with antibiotic resistance.

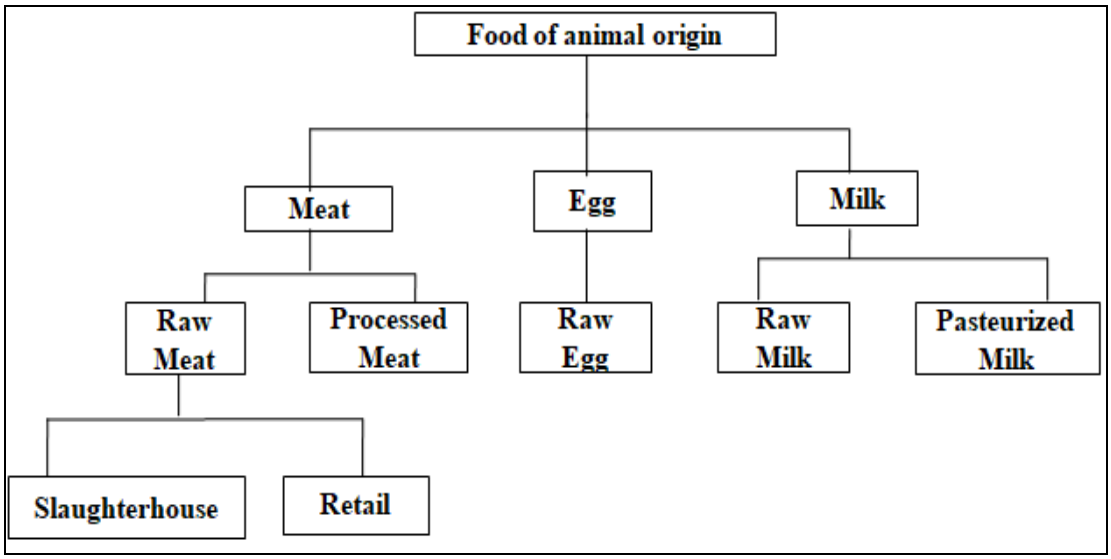

Fig 2.2: Flow chart illustrating types of food samples of animal origin

General considerations:

a. The samples should never be touched with bare hands, and gloves should be used for handling samples.

b. Sterile instruments should be used for cutting, removing and manipulating all samples.

c. The samples should be kept in the proper sterile containers till the next processing step. The outside surface of the container must be sanitized thoroughly.

d. Samples should be weighed directly in the sterile container intended for dilution, mixing, blending and/or stomaching.
e. Clean and disinfect the area after weighing with the same disinfectant that was initially used for disinfecting the work area.
f. Appropriately sterilize the instrument, prior to direct contact with the samples.
g. All containers, gloves and other materials in contact with the sample must be incinerated or terminally sterilized before cleaning or disposal.

2.3.3.1 Meat

Collection and transport

a. All meat samples should be collected in the sterile polythene bags.
b. Cut 100 g meat block from the centre with a sterile knife and place in sterile bag. Use separate sterile plastic bag for each sample.
c. All samples should be transported to the laboratory on ice and processed within 5 hours of receipt at laboratory for bacteriological examination.
d. In case of delay, samples should be refrigerated at 4°C and processing should be done within 96 hours of sampling.

Processing

a. Open the bag and place samples aseptically on a clean surface.
b. Place 25 g (recommended amount) of meat sample into a sterile plastic bag using sterile instruments (e.g. gloves, tongs etc.).
c. Add 225 ml BPW to the bag containing sample (1: 9 ratio).
d. Homogenize it for 3 minutes by manual mixing, or mix on a mechanical shaker at 200 rpm for 15 minutes.
e. Transfer the 50 ml rinse aseptically from the bag into a sterile flask or container.

2.3.3.2 Egg

Collection, transport and Processing

a. The egg samples should be collected aseptically. Samples should be kept in sterile plastic bags and transported to the laboratory.
b. The sample (egg white as well as egg yolk) should be diluted with peptone water (1:1) or pre-enriched with BPW or mannitol salt broth for gram negative or gram-positive bacteria respectively.
c. Isolation of specific pathogens should be performed as mentioned in section 2.4.

2.3.3.3 Milk

Collection and transport

a. Milk samples should be collected aseptically in a jar or container.
b. Samples should be stored on ice during transport to the laboratory while maintaining sterile conditions.

Processing
a. All samples to be kept at 4°C until processed for bacteriological analysis.
b. The sample should be diluted with peptone water (1:1) or pre enriched with BPW for gram negative, and with mannitol salt broth for gram positive bacteria.
c. Isolation of specific pathogens should be performed as mentioned in section 2.4.

2.4 Methods for isolation of bacteria

The compositions of various culture media for the isolation of bacteria are listed in the **Appendix I**.

2.4.1 Enterobacteriaceae (*Escherichia coli* and *Klebsiella*)

a. Serially dilute the swab or faecal suspension in sterile peptone water (10 fold dilutions).
b. Place a loopful of appropriate dilution on MacConkey agar plate, flame the loop and streak from the inoculated area over the entire plate.
c. Incubate the plates aerobically overnight at 37^{o}C and then examine for bacterial growth.
d. Subculture the apparent colonies on nutrient agar plates/slants and incubate at 35°C for 18-24 hours.
e. Perform standard biochemical tests to confirm the isolates as *E. coli* or *Klebsiella* spp.

***E. coli*:** large non-mucoid colonies with even pink color

***Klebsiella*:** large mucoid colonies with pale pink centers and colorless peripheries, or even pink color

2.4.2 Enterobacteriaceae (non-typhoidal *Salmonella* spp.)[7]

a. Dilute the sample (swab/faeces/food) to 1/10th in BPW for non selective pre-enrichment.
b. Incubate at 37^{o}C for 24 hours.
c. Inoculate 1 ml of pre-enrichment culture into Selenite Cysteine broth (SC) or 100 µl into Rappaport-Vassiliadis soy peptone broth (RVS) for selective enrichment.
d. Incubate in SC or RVS at 37°C for 18 hours or 41.5°C for 24 hours respectively.
e. Inoculate enriched samples onto Brilliant Green Agar (BGA) or Xylose Lysine Deoxycholate agar (XLD), and incubate at 37°C for 24 hours.
f. Subculture the suspected colonies on nutrient agar slants/plates and incubate at 37°C for 18-24 hours.

g. Perform standard biochemical tests to confirm the isolates.

BGA: red colonies

XLD: clear colonies with a black center

2.4.3 *Staphylococcus* spp.

a. Dilute 1 ml sample (swab/faecal/food) $1/10^{th}$ in mannitol salt broth and incubate at 37^{o}C for 24 hours for pre-enrichment.

b. Streak a loopful of suspension on Mannitol Salt Agar (MSA) or BPA (Baird Parker Agar) medium supplemented with egg-yolk tellurite emulsion and incubate for 48 hours at 37°C.

c. Subculture suspected colonies on nutrient agar slants and incubate at 37°C for 18-24 hours.

d. Perform standard biochemical tests to confirm the isolates.

BPA: jet black colonies surrounded by a white halo zone

MSA: yellow colonies

Coagulase negative staphylococci **(CoNS) with MSA**: small red to pink colonies

2.4.4 *Enterococcus* spp.

a. Dilute the sample (swab/faecal/milk) $1/10^{th}$ in peptone water.

b. Streak a loopful of suspension on a selective medium Slanetz and Bartley agar (SBA) and incubate at 42°C for 48 hours.

c. Select *Enterococcus* spp. colonies based on the colony morphology (typical shape and color).

E. faecium: large colonies with a red centre and distinct white edge

E. faecalis: red to deep maroon centre

E. hirae: small dark maroon colonies

E. casseliflavus: pale pink

Identification of Isolates to Species Level

CHAPTER 3

IDENTIFICATION OF ISOLATES TO SPECIES LEVEL

3.1 Biochemical tests for identification

3.1.1. Identification of Enterobacteriaceae (*E. coli* and *Klebsiella*)

Following tests should be used for the biochemical characterization of commonly isolated members of family Enterobacteriaceae.

Table 3.1: Biochemical test for identification of *E.coli* and *Klebsiella*

Bacteria	Lactose	Motility	Gas	Indole	VP	Citrate	PDA	Urease	Lysine	H_2S	Inositol	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+

Lactose and inositol indicate fermentation of lactose and inositol; gas indicates gas from glucose; citrate indicates citrate utilization (Simmons); PDA indicates phenylalanine deaminase; lysine indicates lysine decarboxylase; H_2S indicates H_2S production in triple sugar iron (TSI) agar; ONPG indicates metabolism of o-nitrophenyl-β-D-galactopyranoside; VP: Voges-Proskauer; + indicates $\geq$ 85% of strains positive; - indicates $\geq$ 85% strains negative

3.1.2 Non-typhoidal *Salmonella* (NTS) [*S. enteritidis, S. typhimurium, S. pullorum, S. gallinarum* etc.]

3.1.2.1 Morphology and cultural characteristics

NTS are gram negative rod-shape, non-spore-forming, and predominantly motile by means of peritrichous flagella. Colonies are generally lactose non-fermenters. Isolates are identified by a combination of colonial appearance, serology (agglutination with specific antisera) and biochemical testing. After growth on selective media for 48 hours, *S. pullorum* grows slowly and produce very small colonies; however, *S. gallinarum* grows more rapidly than *S. pullorum* and produces larger colonies with a distinctive smell resembling that of seminal fluid on most of the media.

Table 3.2: Criteria for biochemical characterization of non-typhoidal *Salmonella* (NTS)

	S. enterica (most serovars, NTS)	*S. pullorum*	*S. gallinarum*
TSI butt	A	A	A
TSI H2S	+	V	V
TSI gas	+	+	-
Lysine decarboxylation	+	+	+
Motility	+	-	-
Ornithine decarboxylation	+	+	-
Urea	-	-	-
Citrate	+	-	-
Dulcitol	+	-	+
Maltose fermentation	+	– or late +	+
Gas from glucose (medium with Durham's tube)	+	+	-

K- Alkaline; A- Acid; V- variable; + indicates ≥ 85% of strains positive; - indicates ≥ 85% strains negative.

3.1.2.2 Agglutination tests for *Salmonella* spp.[8]

The following limited ranges of antisera are adequate for routine use in slide agglutination test for serological identification:

- Polyvalent O
- Single factor O (2, 4, 6, 7, 8, 9, 3, 10)
- Polyvalent H
- Rapid H sera (RSD 1, 2, 3)
- Polyvalent H phase 2 (1-7)
- Single factor H (a, b, c, d, E, G, i, r)

Method

a. Select five suspect colonies from non-selective media (nutrient or blood agar).

b. Take a drop of saline on a clean slide. Make a smooth suspension of the growth and check for absence of auto-agglutination.

c. Add a drop of the required antisera and mix thoroughly. Look for agglutination within 30 seconds.

d. Gently rock the slide or stir the drop with a loop for 30-60 seconds, and observe for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope.

e. Test positive (clumping) isolates with poly O and poly H antisera first. If positive, then test for other groups starting from A-G groups as per specific organism.

f. *S. pullorum* and *S. gallinarum* should agglutinate with polyvalent 'O' antisera, but not with polyvalent flagella (poly 'H' phase 1 and phase 2) antisera.

g. If poly 'O' is positive then check with 'O'9 antiserum. If 'O'9 is positive and poly 'H' is negative, it indicates the possible presence of *S. pullorum or S. gallinarum*.

h. Confirm *S. pullorum* and *S. gallinarum* using biochemical tests.

3.1.3 Identification of *Staphylococcus*

Morphology and cultural characteristics

Members of genus *Staphylococcus* are gram positive cocci that can form in grape like clusters. They are facultative anaerobic organisms. Species of this genus are both coagulase-positive and coagulase-negative.

Perform biochemical identification and characterization of *Staphylococcus* isolates after confirmation of gram reaction and catalase test.

Colonies characterization of *Staphylococcus* and CoNS on different media as below:

BPA: jet black colonies surrounded by a white halo zone

MSA: yellow colonies

Coagulase negative staphylococci (CoNS) on MSA: small red to pink colonies

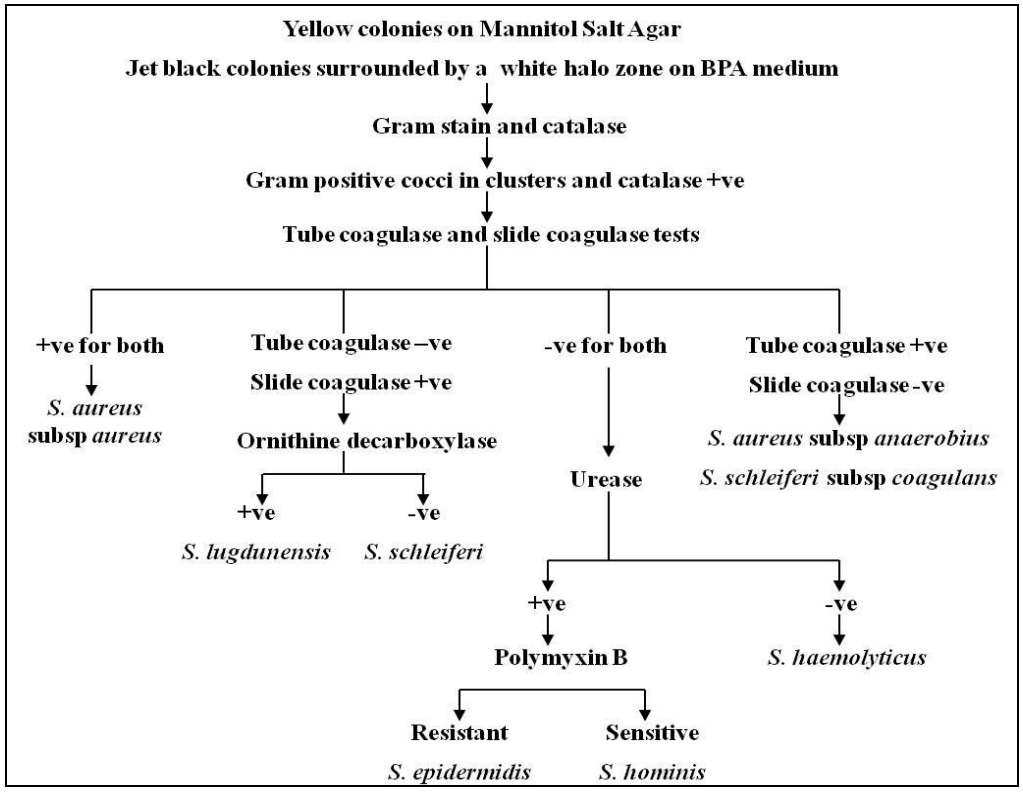

Figure 3.1: Biochemical characterization for speciation of Staphylococcus isolates

3.1.4 Identification of Enterococcus

3.1.4.1 Morphology and cultural characteristics

Members of the genus *Enterococcus* are catalase-negative, gram-positive cocci that can occur either as pairs or in the form of short chains. After growth on blood agar for 24 hours, most of the isolates of *Enterococcus* show α-hemolysis or γ-hemolysis on sheep blood agar, although some strains may be β-hemolytic. About a third of the isolates of *Enterococcus* show β-hemolysis on blood agar containing human blood or rabbit blood.

3.1.4.2 Identification

- Preliminary identification of genus *Enterococcus* is made on the basis of tests like hydrolysis of bile-esculin, growth in 6.5% NaCl broth and heat tolerance. However, some other genera of catalase negative bacteria like *Leuconostoc*, *Pediococcus* and *Vagococcus* resemble *Enterococcus* in some phenotypic characteristics.
- Few tests like PYR (L-pyrollidonyl β-naphthylamide) test and detection of Lancefield's group D antigen can help to distinguish *Enterococcus* from other genera.

3.1.4.3 Speciation of *Enterococcus*

The most widely used identification and characterization scheme for *Enterococcus* species is the 'Facklam and Collins identification scheme'. However, most of the frequently isolated species of *Enterococcus* can be identified by using simpler identification schemes using lesser number of biochemical tests (Fig 3.2).

3.1.4.4 Group categorization of *Enterococcus*

Enterococcus is categorized into 5 groups for easier characterization and identification on the basis of simplified biochemical tests like acid production from mannitol and sorbose and dihydrolysis of arginine.

Table 3.3: Group categorization of *Enterococcus*

Group I	*E. avium, E. raffinosus, E. pallens, E. malodoratus*
Group II	*E. faecalis, E. faecium, E. casseliflavus, E. gallinarum, E. mundtii*
Group III	*E. dispar, E. hirae, E. durans*
Group IV	*E. cecorum, E. asini, E. phoeniculicola*
GroupV	*E. canis, E. columbae*

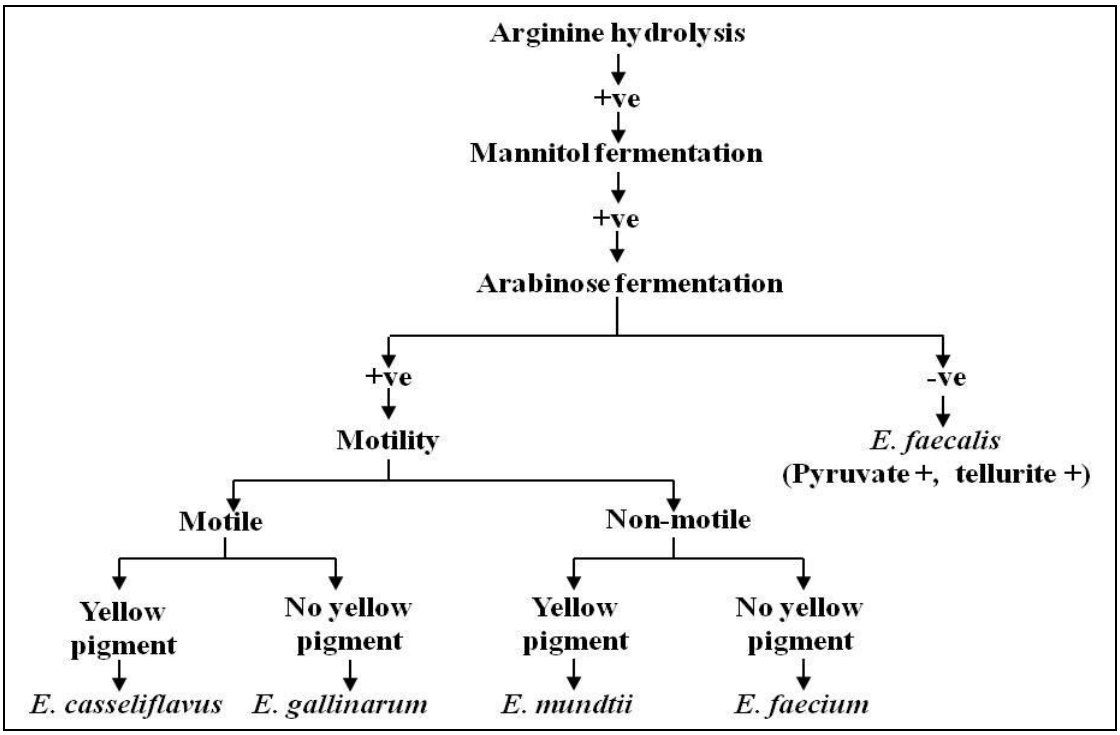

Figure 3.2: Phenotypic speciation of Enterococcus isolates

3.2 Molecular tests for identification

Isolation of genomic DNA

DNA may be extracted from overnight cultures grown on BHI agar at 37°C using manual method or with high quality kit as per manufacturer's instructions. The concentration of genomic DNA should be determined and it should be store at −20°C until use.

3.2.1 *E. coli*

Perform multiplex PCR to target five housekeeping genes in suspected *E. coli* colonies: ***uid*A** (ß-D-galactosidase), ***lac*Z** (ß-D-galactosidase), ***lac*Y** (lactose permease), ***cyd*** (cytochrome bd complex) and ***pho*A** (bacterial alkaline phosphatase).

3.2.1.1 PCR program

Pre-denaturation step of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds, and final extension step of 72°C for 5 minutes.

Table 3.4: Primer for specific gene detection of *E. coli*

Gene	Sequence (5'-3')	Concentration (µM)	Amplicon size (bp)	References
Lac Y	'F' CTACCGGTGAACAGGGTAGC 'R' GTCGCTGAAAAACGCACTTC	0.15	289	9
LacZ	'F' ATGAAAGCTGGCTACAGGAAGG 'R' CTCCACACAGTT TCGGGTTTTC	0.5	517	9
Cyd A	'F' CCGTATCATGGTGGCGTGTGG 'R' GCCGGCTGAGTAGTCGTGGAAG	0.4	398	9
Uid A	'F' CGCCGATGCAGATATTCG 'R' GCTGTGACGCACAGTTCATAG	0.4	603	9
Pho A	'F' GGTAACGTTTCACCGCAGAGTTG 'R' CAGGGTTGGTACACTGTCATTACG	0.6	468	9

If amplicons of above mentioned four or five genes obtained, then isolate should be confirmed as *E. coli* otherwise (less than four genes amplicons) isolate should be recorded as non-*E. coli*.

3.2.2 *Klebsiella* spp.

gyr*A** gene may be targeted for genus level identification of *Klebsiella* spp. Species level identification of *K. pneumoniae* and *K. oxytoca* may be carried out by targeting ***rpoB and ***pehX*** genes respectively.

Table 3.5: Primer for specific gene detection of *Klebsiella*

Microorganism	Target genes	Sequences(5'-3')	Amplicon size (bp)	Ref.
Klebsiella spp.	*gyr A*	F' CGCGTACTATACGCCATGAACGTA R' ACCGTTGATCACTTCGGTCAGG	441	10
K. pneumoniae	*rpo B*	F' CAACGGTGTGGTTACTGACG R' TCTACGAAGTGGCCGTTTTC	108	11
K. oxytoca	*peh X*	F' GATACGGAG TATGCCTTTACGGTG R' TAGCCTTTATCAAGCGGATACTGG	343	11

3.2.2.1 PCR program

*gyr*A gene *(Klebsiella* spp.): Initial denaturation of 95 °C for 15 minutes; 35 cycles of 95 °C for 1 minute (denaturation), 55 °C for 1 minute (annealing), 72 °C for 2 minutes (extension); and final extension of 72 °C for 5 minutes.

*rpo*B gene (*K. pneumoniae)* and *pehX gene (K. oxytoca*): Initial denaturation of 95 °C for 10 minutes; 35 cycles of 95 °C for 1 minute (denaturation), 55 °C for 1 minute (annealing), 72 °C for 1 minute (extension); and final extension of 72 °C for 5 minutes.

3.2.3 *Salmonella* spp.

Genus level identification of *Salmonella* spp. can be carried out using following primers to target ***stn*** gene with a PCR program of initial denaturation of 95 °C for 3 minutes; 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72°C for 30 seconds; and final extension of 72 °C for 5 minutes.

Table 3.6: Primer for specific gene detection of *Salmonella*

Salmonella enterica	Target genes	Sequences(5'-3')	Amplicon size (bp)	Reference
	stn	F' CTTTGGTCGTAAAATAAGGCG R' TGCCCAAAGCAGAGAGATTC	260	12

3.2.4 *Staphylococcus* spp.

Firstly, *Staphylococcus* isolate may be confirmed using genus specific PCR.

Table 3.7: Primer for genus specific PCR for *Staphylococcus* isolate confirmation

Gene	Sequence (5'-3')	Annealing Temp (°C)	Amplicon size (bp)	Reference
16S rRNA	'F' GTGATCGGCCACACTGGA 'R' CAACTTAATGATGGCAACTAAGC	60	842	13

In genus positive isolates, identify *Staphylococcus* species through pentaplex PCR. If pentaplex PCR is negative, confirm *Staphylococcus* spp. isolates through 16S *rRNA* sequencing by Basic Local Alignment Search Tool (BLAST) analysis. In case of uncertainty, PCR should be carried out using species specific primers to accurately confirm the species identity.

Table 3.8: Primer for specific gene detection of *Staphylococcus*

Staphylococcus species	Gene	Sequence (5'-3')	Annealing Temp (°C)	Amplicon Size (bp)	Reference
S. aureus	23S rRNA	F' AGCGAGTCTGAATAGGGCGTTT R' CCCATCACAGCTCAGCCTTAAC	56	894	13,14
S. chromogenes	*sodA*	F'GCGTACCAGAAGATAAACAAACTC R'CATTATTTACAACGAGCCATGC	58	222	13,14
S. haemolyticus	*sodA*	F'CAAATTAAATTCTGCAGTTGAGG R'GGCCTCTTATAGAGACCACATGTTA	58	531	13,14
S. epidermidis	*rdr*	F'AAGAGCGTGGAGAAAAGTATCAAG R'TCGATACCATCAAAAAGTTGG	56	130	13,14

S. scuiri	*gap*	F'GATTCCGCGTAAACGGTAGAG R'CATCATTTAATACTTTAGCCATTG	56	306	13,14
Staphylococcus spp.	16S *rRNA*	F'GCGGACGGGTGAGTAACAC R'GACGACAACCATGCACCAC	60	974	13,14

Table 3.9: Identification of other *Staphylococcus* spp. through species specific PCR

Staphylococcus species	Gene	Sequence (5'-3')	Annealing temp (°C)	Amplicon size (bp)	Reference
S. hyicus	*sod*A	F'TATTGAAGAGCTTATCGCGAATGT R'ATCGTGCTGCTGCTTTATCTGAG	58	215	13
S. simulans	*gap*	F'AGCTTCGTTTACTTCTTCGATTGT R'AAAAGCACAAGCTCACATTGAC	60	472	13
S. auricularis	*gap*	F'TGCAAGGTCGTTTCACAAGT R'TGTACCATCAAGTGTATCGTGGT	60	278	13
S. hominis	*sodA*	F'TTTTAAGCAAGACAATCGACCTCA R'CCAAATTTACCATATGCAGCAG	60	727	13
S. cohnii	*gap*	F'CGGCTTAATCGAAGGTTTCA R'GATGCATTTTTCATTGCGTTA	58	297	13
S. xylosus	*16-23S ISR*	F'GGATATATTCGGAACATCTTCTTTA R'TTTTTGACTTTTAACACGACGAAG	56	280	13

ISR: Intergenic Spacer Region

3.2.5 Enterococcus spp.

Genus level identification of *Enterococcus* spp. can be carried out using genus specific primers that target **16SrRNA** gene. Further, species level identification of *E. faecalis* and *E. faecium* may be performed by targeting ***ddl*** genes[15].

Table 3.10: Primer for specific gene detection of *Enterococcus spp.*

	Target genes	Sequences (5'-3')	PCR program	Amplicon size(bp)	Ref
Enterococcus spp.	*16S rRNA*	F' GACTACCNGGGTATCTAATCC R' AGAGTTTGATCCTGGCTNAG	Initial denaturation of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 90 seconds, and 72°C for 60 seconds; Final extension of 72° C for 10 minutes	800	15
E. faecalis	*ddl*	F' ATCAAGTACAGTTAGTCTT R' ACGATTCAAAGCTAACTG		941	15
E. faecium	*ddl*	F' TAGAGACATTGAATATGCC R' TCGAATGTGCTACAATC		550	15

Antimicrobial Susceptibility Testing

CHAPTER 4

ANTIMICROBIAL SUSCEPTIBILITY TESTING

All the participating centers have to follow CLSI 'Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals' for antimicrobial susceptibility testing[16].

4.1 Definitions

4.1.1 Breakpoint (BP) - zone diameter or minimal inhibitory concentration (MIC) value used to indicate susceptible, intermediate, and resistant.

4.1.2 Susceptible (S) - a category defined by a breakpoint that implies that isolates with an MIC at or below or zone diameters at or above the susceptible breakpoint are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used, resulting in likely clinical efficacy.

4.1.3 Intermediate (I) - defined by a breakpoint that includes isolates with MICs or zone diameters within the intermediate range that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. It implies:

a) Clinical efficacy in body sites for which the drugs are physiologically concentrated or when a higher than normal dosage of a drug can be used.

b) A buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

4.1.4 Resistant (R) - defined by a breakpoint that implies that isolates with an MIC at or above or zone diameters at or below the resistant breakpoint are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs or zone diameters that fall in the range in which specific microbial resistance mechanisms are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in isolates with similar phenotypes.

4.1.5 Nonsusceptible (NS) - isolates for which only a susceptible breakpoint is designated because of the absence or rare occurrence of resistant strains. Isolates for which MICs of antimicrobial agents are above or zone diameters are below the value indicated for the susceptible breakpoint should be reported as nonsusceptible.
Note: An isolate interpreted as nonsusceptible does not necessarily mean that the isolate

has a resistance mechanism. The term “nonsusceptible” should not be used when describing an organism/drug category with intermediate and resistant interpretive categories. Isolates that are in the categories of “intermediate” or “resistant” could be called “not susceptible” rather than “nonsusceptible.”

4.1.6 Minimum inhibitory concentration (MIC) -lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

4.1.7 Prevention/prophylaxis - administration of an antimicrobial agent to exposed apparently healthy animals considered at risk, but before onset of disease, and for which no etiological agent has yet been confirmed by culture or other detection method(s); NOTE: Generally used in a herd or flock situation and not an individual animal.

4.1.8 Production use - administration of an antimicrobial agent, usually as medicated feed over a period of time to growing animals that results in improved physiological performance (eg. for increased rate of weight gain and improved feed efficiency), sometimes referred to as growth promotion, health maintenance, or non-therapeutic use.

4.1.9 Treatment - administration of an antimicrobial agent to an animal or group of animals that exhibits overt clinical disease.

4.2 Considerations for performing susceptibility testing

- Isolated colonies from specimens of animals for each type of microorganism that may play a pathogenic role should be selected from primary (nonselective) agar plates.
- Mixtures of different types of organisms should not be tested on the same susceptibility test plate. In case of mixed cultures, isolated colonies may be obtained after overnight subculture with proper streaking.
- The susceptibility testing directly with the clinical material should be avoided except in clinical emergencies when direct gram stain shows a single pathogen. The result should be reported as preliminary, and the susceptibility must be repeated using standard methodology.

4.2.1 Disk Diffusion Susceptibility Testing

4.2.1.1 Recommended media

Mueller Hinton agar (MHA): Use the fresh plates same day or store in a refrigerator (2-8ºC), wrap the plates in plastic before refrigerating to minimize the evaporation. If excess moisture is visible on the surface before use, place the plates in an incubator (35ºC) or in a laminar-flow hood with lids ajar at room temperature until the moisture evaporates (usually 10-30 minutes).

4.2.1.2 Preparation of inoculum

Direct colony suspension method

Prepare the inoculum by making a direct saline or broth suspension of colonies from an overnight incubated agar plate (nonselective medium such as blood agar/chocolate agar) to obtain the 0.5 McFarland turbidity (1-2 x 10^8 CFU/ml) of *E. coli* (ATCC® 25922). This is the method of choice for testing species that fail to grow satisfactorily in broth media (i.e. *Staphylococcus* spp. for methicillin resistance).

Broth culture (growth) method

Choose at least four to five well-isolated colonies of the same morphological type from an overnight incubated nonselective agar plate. With the sterile loop or straight wire, touch the top of each colony and transfer the growth to a tube containing 4-5 ml of a suitable broth medium, such as tryptic soy broth. Incubate tube at 35ºC till turbidity of 0.5 McFarland tube or more is achieved. Adjust the turbidity of culture with sterile saline or broth to exactly 0.5 McFarland turbidity standard. Use of the spectrophotometric method is preferable over visual determination with Brown's tubes. For visual comparison, look through the broths in transmitted light against a white background with contrasting black stripes.

4.2.1.3 Inoculation of test plates

Inoculate MHA (Mueller-Hinton agar) plate within 15 minutes after adjusting the inoculum suspension. Dip a sterile nontoxic cotton into the suspension, rotate several times, and gently press onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. Streak the swab over the entire surface of the agar plate three times, with rotating the plate approximately 60 ºC each time to ensure even distribution of the inoculum. A final sweep of the swab will be made around the agar rim. The lid may be left ajar for 3 to 5 minutes but no longer than 15 minutes to allow the absorption of any excess surface moisture before applying the drug-impregnated disks.

4.2.1.4 Application of antimicrobial disks to an agar plate

This step should be done within 15 minutes of inoculation of test plates. Dispense the selected antimicrobial disks evenly onto the agar plate with the help of a sterile forceps/ needle tip. Flame the tip of the applicator intermittently. Gently press down each disk to ensure complete contact with the agar surface.

i. No more than 12 disks should be applied on a 150 mm plate or 5 disks on a 100 mm plate, keeping at least a distance of 24 mm between disks. For fastidious organisms, no more than 9 disks should be applied to the surface of a 150 mm plate or no more than 4 disks on a 100 mm plate. Avoid dispensing disks too near to the edge of the

plate. A disk must not be relocated once it has come in contact with the agar surface because some of the drugs diffuse instantaneously.

ii. It is advisable to place disks that give predictably small zones (e.g. aminoglycosides) next to those disks that give larger zones (e.g. cephalosporins) to avoid overlapping of zones.

iii. Remove the disk containers from the refrigerator or freezer, 1-2 hours before use to equilibrate disks to room temperature before opening. This step minimizes the amount of condensation that occurs when warm air contacts cold disks.

Note: Sealed packages of the disks that contain drugs from the β-lactam class should be stored frozen, except for a small working supply, which may be refrigerated for one week at most. Some labile agents (e.g. imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.

iv. Use only those disks that have not reached the manufacturer's expiration date as stated on the label. Unused disks should be discarded upon the expiration date.

4.2.1.5 Incubation

Invert the plates and incubate at 35 ± 2 ºC within 15 minutes in ambient air after disks application. For fastidious organisms, incubate plates at 35 ± 2 ºC in 5 ± 2% CO_2. If a CO_2 incubator is not available, a candle extinction jar is an acceptable alternative.

4.2.1.6 Reading of plates

Examine each plate after overnight incubation 16-18 hours (fastidious organisms 20-24 hours) for confluent growth and circular zones of inhibition. Measure the diameters of the zones of complete inhibition including the diameter of the disk, to the nearest whole millimetre (mm) using callipers or a ruler. Consider the area as end point (margin) of zone that shows no obvious visible growth, detectable with the unaided eye. Ignore faint growth of tiny colonies visible only with the magnifying lens.

i. With unsupplemented MHA plate, hold the measuring device on the back of inverted petri dish which is illuminated with reflected light against a black, nonreflecting background.

ii. For opaque media like MHA supplemented with blood, measure zone sizes from the upper inoculated surface with cover removed and illuminated with reflected light.

iii. In case of presence of discrete colonies within clear zone of inhibition, repeat test with a pure culture or subculture of a single colony from the primary culture plate. If discrete colonies still appear, measure the obvious outline zone of inhibition.

iv. Ignore swarming of *Proteus* spp.

v. With trimethoprim, sulfonamides, and the combinations of two agents, antagonists in the medium may allow some minimal growth; therefore, measure the obvious margin of zone diameter and disregard slight growth (20% or less of the lawn of growth).

Notes:

a) For coagulase-negative staphylococci (CoNS) with Cefoxitin, 24 hours of incubation are needed before reporting as susceptible. Read and report other agents at 16-18 hours. If Cefoxitin is tested against *Staphylococcus* spp., read the zone diameters with reflected, not transmitted light (plate held up to the light).
b) If testing Vancomycin against *Enterococcus* spp., 24 hours of incubation are needed before reporting as susceptible. Read and report other agents at 16-18 hours. Read the zone diameters with transmitted light for Vancomycin against *Enterococcus* spp.
c) For *S. aureus* strains with penicillin zones ≥ 29 mm, interpret the appearance of zone positive for sharp zone edge (cliff) or negative for fuzzy zone edge (beach).
d) Vancomycin disk diffusion testing is not recommended for *S. aureus* or CoNS.

4.2.1.7 Interpretation and reporting of results

Interpret the sizes of the zones of inhibition by referring to tables (Chapter AST and MIC Breakpoints), and report the organism as susceptible, intermediate, or resistant to each antimicrobial agent tested.

Before reporting results, special consideration must be given to:

- Unexpected or unusual phenotype resistance
- Additional species-specific and screening tests to detect resistance
- Evaluation of QC results

4.3 Preparing antibiotic disks in-house

4.3.1 Method

a. Use appropriate solvents and diluents for the preparation of stock solutions of antimicrobial agents. The molarity and pH of various diluents are listed in the **Appendix II.** In most cases it is sterile distilled water (DW) or phosphate buffered saline (PBS), pH 7.2.
b. On the vial of the antibiotic powder, read the potency of the antibiotic, amount, and its value in international units, if any.
c. Find out the amount (e.g. µg/disk or units/disk) of antibiotic mentioned for a single disk[17]. Apply proper conversion if antibiotic powder is labeled in units and assayed in milligrams.
d. The amount mentioned per disk is to be dissolved in 10 µl of the solvent. Adding correction for the potency, calculate how much is to be dissolved in say, 4 ml.
e. Now take 4 ml of the sterile solvent in a test tube.
f. Measure the exact amount of antimicrobial agent to be added to 4 ml of the solvent. Use the milligram balance, and aluminum foils (sterile, if possible) to weigh the antimicrobial.

g. Now dissolve antimicrobial in the solvent (depending on the solubility, it may take seconds to an hour or more). If it does not dissolve fast, keep the test tube in the incubator and shake intermittently.

h. Prepare disks approximately 5-8 mm in diameter from Whatman filter paper no. 3. Place the disks in a petri dish and sterilize in a hot air oven.

i. Work in a biosafety hood from this step. Dispense sterile disks (400 for 4 ml of antimicrobial solution) onto pre-sterilized petri plates. Keep the disks apart, do not touch the disks; use a forceps to separate the disks. Flame the tip of the forceps intermittently.

j. Add exactly 10 µl of the antibiotic solution to each disk. Once finished, leave the petri-dish slightly open in the incubator for 30 minutes to 1 hour to allow the drying of disks.

k. When dry, use the forceps to transfer the disks to an appropriate container. A small pack of silica gel may be added to keep moisture away.

l. Store disks at 4 ºC. For degradable antimicrobials like clavulanic acid and imipenem, store at -20 ºC and take these out only just before use.

4.3.2 Storage of antimicrobial disks

Cartridges containing commercially prepared antibiotic paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks should be stored at 8 °C or below or frozen at -14 °C or below, in a non frost-free freezer until needed. Sealed packages of disks that contain drugs from the ß-lactam class should be stored frozen (-20 ºC) except for a small working supply which may be refrigerated for at most one week. Some labile agents (e.g. imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.

Minimum Inhibitory Concentration (MIC) Testing

CHAPTER 5

MINIMUM INHIBITORY CONCENTRATION (MIC) TESTING

Both broth and agar dilution techniques may be used to measure quantitatively the *in vitro* activity of an antimicrobial agent against a given bacterial culture. Prepare a series of tubes, wells, or plates with a broth or agar medium to which various concentrations of the antimicrobial agents are added. Then, inoculate the tubes, wells, or plates with a suitably standardized suspension of the test organism. After overnight incubation at 35°C, examine the tests and determine the MIC.

5.1 Broth dilution method[18]

5.1.1 Broth macrodilution (tube) MIC testing

Several sterile tubes will be arranged and labeled in the range of MICs to the particular antimicrobial under consideration.

5.1.1.1 Inoculum

i. To get pure culture of target organism, suspend either 3-5 colonies in normal saline or use the growth method as described previously to get an inoculum of 0.5 McFarland (1.5×10^8 cfu/ml for *E. coli* ATCC® 25922).
ii. Make 1 in 100 dilution of the suspension by adding 0.1 ml growth into 9.9 ml normal saline (~ 10^6 cfu/ml).
iii. Final inoculum concentration will be 3-5 x 10^5cfu/ml after adding 0.25 ml of the above suspension into each tube containing broth.

5.1.1.2 Media

Add 0.5 ml of double strength Mueller Hinton broth (DS MHB) into each tube. For oxacillin MIC of *Staphylococci*, use DS MHB with 2-4% NaCl.
Note: MHB should contain 20-25 mg/L of Ca^{++} ions and 10-12.5 mg/L of Mg^{++} ions.

5.1.1.3 Antibiotic dilutions

The concentrations tested for a particular antimicrobial agent should include the breakpoints of that antibiotic. For serial twofold dilution MICs, the dilution scheme should be used as follows: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, etc. It is advised to choose a MIC range that allows at least one QC organism to have on-scale values.

1) To prepare the 4X stock solutions of the antibiotic as follows:

a) Dissolve 10 mg of the desired antibiotic in the appropriate diluent (mostly DW). Also take into consideration the potency of the antibiotic as specified by the manufacturer

and obtain a master-stock solution (label as master-stock 1) of 10 mg/ml (10,000 µg/ml) of active salt.

b) Next, prepare two more master-stock solutions of 1 mg/ml (1000 µg/ml; master-stock 2), and 100 µg/ml (master-stock 3) by serial 10 fold dilutions (1 ml in 9 ml DW).

c) Label sterile tubes as 4X antibiotic solution of the desired concentration range (512 µg/ml to 0.25 µg/ml). Prepare 4X stock solutions of concentrations 0.25-512 µg/ml for a test range of 0.0625-128 µg/ml, from the master stocks.

d) In distinctly labeled tubes, add 102.4 µl, 51.2 µl, 25.6 µl and 12.8 µl of stock 1 (10,000 µg/ml) to get antibiotic concentration 512 µg/ml, 256 µg/ml, 128 µg/ml, and 64 µg/ml respectively.

e) Similarly add 64 µl, 32 µl, and 16 µl of stock 2 (1000 µg/ml) and 80 µl, 40 µl, 20 µl, 10 µl and 5 µl of stock 3 (100 µg/ml) to get solutions of test range 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1.0 µg/ml, and 0.5 µg/ml. Make final volume 2 ml in each tube with diluent. These are the 4X stock solutions.

2) Label another set of test tubes and add 0.25 ml of the 4x antibiotic solution to each of the specified tubes. Make final tube volume 1 ml for desired test concentration range (Table 5.1).

Table 5.1: Method of broth macrodilution MIC testing

Sr. No.	Master stock	Volume of antibiotic master-stock (µl)	Final volume (ml)	4X stock solution (µg/ml)	Volume of 4X stock (ml)	Final volume (ml)	Test range (µg/ml)
1.	1	102.4	2 ml with diluent in each tube	512	0.25	1 ml with diluent in each tube	128
2.	1	51.2		256	0.25		64
3.	1	25.6		128	0.25		32
4.	1	12.8		64	0.25		16
5.	2	64		32	0.25		8
6.	2	32		16	0.25		4
7.	2	16		8	0.25		2
8.	3	80		4	0.25		1
9.	3	40		2	0.25		0.5
10.	3	20		1	0.25		0.25
11.	3	10		0.5	0.25		0.125
12.	3	5		0.25	0.25		0.0625

5.1.1.4 Controls: Use one growth control tube containing no antibiotic; use one sterility control tube (negative control) without any inoculum. Also test one standard strain as test control.

5.1.1.5 Incubation: 35-37 ºC

5.1.1.6 Period of incubation: 16-20 hours except for oxacillin for *Staphylococcus,* and vancomycin for *Enterococcus* where it should be 24 hours. To ensure the detection of methicillin and vancomycin resistance, the direct inoculum method is recommended, and incubation should be for a full 24 hours.

5.1.1.6 Reading: MIC is the lowest concentration of antibiotic at which there is no visible growth of the microorganism in the tubes.

5.1.2 Broth MIC testing

Use sterile, plastic microdilution trays that have round or conical bottom wells and double strength Mueller Hinton broth (DS MHB). Dispense 0.1 ml of broth in each well. Make the antimicrobial dilutions as described above and dispense 0.1 (± 0.02) ml from each tube into the respective wells. Also, keep growth control wells containing no antibiotic in each set. Seal the filled trays in plastic bags and immediately place in a freezer at −70 °C or colder until needed.

Note: If antibiotics need to be used for reference work, use within four weeks after preparation. Do not refreeze thawed solutions nor store trays in a self-defrosting freezer.

5.1.2.1 Inoculum

Prepare inoculum as stated in macrodilution testing. Dilute the turbidity adjusted culture in broth and inoculate (5×10^5 CFU/mL) within 15 minutes. Seal each tray in a plastic bag with plastic tape, or with a tight-fitting plastic cover before incubation to prevent drying.

Note: If the volume of the inoculum exceeds 10% of the volume of the well, the diluting effect of the inoculum on the antimicrobial agent must be taken into account.

5.1.2.2 Incubation: 35-37 ºC (Do not stack microdilution trays more than four high to maintain the same incubation temperature for all cultures)

5.1.2.3 Period of incubation: 16-20 hours except for oxacillin for *Staphylococcus,* and vancomycin for *Enterococcus* where it will be 24 hours.

5.1.2.4 Reading: Compare the amount of growth in the wells containing antibiotic with the growth-control wells (no antimicrobial agent) to determine the growth end points. For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth- control well.

5.1.3 Broth dilution automated MIC testing

Sensitive optical detection systems allow the detection of subtle changes in bacterial growth and often produce susceptibility test results in a shorter period than manual readings.

5.1.3.1 Identification and antibiotic sensitivity by Vitek
(Note: The example of VITEK2 Compact is being used as an illustrative example)

Purpose: This protocol describes the procedures for identification and susceptibility test of microorganisms using the VITEK 2 Compact Instrument. The V2C is an automated identification system for microorganisms. It is used for organism identification and sensitivity.

Principle of Test: The VITEK 2 is an automated microbiology system utilizing growth-based technology. These systems accommodate the colorimetric reagent cards for organism identification and colometry based antimicrobial susceptibility using MIC test cards that are incubated and interpreted automatically.

Type of primary sample: Culture requirements table that lists parameters for appropriate culture and inoculum preparation. These parameters include acceptable culture media, culture age, incubation conditions, and inoculum turbidity.

Equipment: VITEK 2 Compact Densicheck Plus (bioMerieux Inc. DensiChek User's Manual.)

Reagents

Reagent Cards: The reagent cards have 64 wells that can each contain an individual test substrate for identification and antibiotics with different concentration for sensitivity. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a preinserted transfer tube used for inoculation. Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample after loading the card onto the system (bioMerieux Inc. Vitek 2 Compact Hardware User Manual)

There are currently four reagent cards available for the identification of different organism classes as follows:

1. GN (21341) - Gram-negative fermenting and non-fermenting bacilli
2. GP (21342) - Gram-positive cocci and non-spore-forming bacilli
3. YST (21343) - yeasts and yeast-like organisms
4. BCL - Gram-positive spore-forming bacilli

There are currently four reagent cards available for the sensitivity of different organism classes as follows:

1. AST N281 (414532) - Sensitivity of Gram Negative bacilli
2. AST P628 (414534) - Sensitivity of Gram Positive Cocci
3. AST YS08 (420739) - Sensitivity for Yeast
4. Sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0)

Procedure

Suspension Preparation: A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted accordingly and measured using a turbidity meter called the DensiChekTM. Product McFarland turbidity range follows as:

GN	0.50- 0.63
GP	0.50- 0.63
YST	1.80- 2.20
BCL	1.80- 2.20

Microorganism suspensions from each test tube are sub-cultured on CLED agar media for checking the purity of corresponding suspension tube.

Inoculation: Reagent cards are inoculated with microorganism suspensions. A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 10 tests the filled cassette is placed manually in to incubation chamber.

Card sealing and incubation: Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 60 cards. All card types are incubated on-line at 35.5 + 1.0 ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data are collected at 15-minute intervals during the entire incubation period.

The VITEK system analyses the data results and determines the identification and sensitivity of the test organism. Result data is automatically recorded and generated by the computer in the form of a printout. The printout for the identification and sensitivity of the test organism will be filed in the VITEK 2 Compact Software.

Quality control: Quality control organisms and their expected results are listed for each product and are tested according to the procedures outlined in the on-line product information. Frequency of testing is once every week using all the cards in use. For AST- YST QC is done once every month and /or along with each test run. This is because of the low frequency of yeast isolates.

The laboratory performs antimicrobial susceptibility testing by Vitek 2 compact. The antibiotic panels provided in the cards are fixed and are to be edited in the final result to suit organism and or site of infection in the following manner-

1. Gram Positive Cocci (*Staphylococcus, Enterococcus*): Exclude Nitrofurantoin in all isolates except those from urine.
2. Gram Negative Bacilli (*E. coli*, etc)

- Do not report Nitrofurantoin in any isolate except those from urine.
- Do not report Ertapenem in Non-Fermenters

5.1.3.2 Standardization of DensiCHEKTM Plus using DensiCHEKTM Plus standard kit

Background: DensiCHEKTM plus standards are used to verify the DensiCHEKTM plus instrument measurement performance. Use of three standards enables the accuracy of the instrument to be monitored and therefore confirm accuracy of the organism suspension.
Principle: The true value expressed in McFarland (McF) is printed on the label of the standard tube. Place the standard tube into the DensiCHEKTM plus, the value displayed on the instrument can be compared to the value printed on the label.
Kit content: Set of four standards 0.0 (blank), 0.5 McF, 2.0 McF, and 3.0 McF
Precautions: Do not open the tubes and do not shake any of the standard tubes, since air bubbles can affect readings.
Storage conditions: Do not freeze or over-heat. The kit can be used till the expiration date indicated on the pack.
Temperature: Storage: 5ºC to 30ºC (41ºF to 86ºF); Operating: 15ºC to 30ºC (59ºF to 86ºF)
Relative humidity: Storage: 20% to 85% non-condensing; Operating: 20% to 80% non-condensing
Test frequency: It is recommended to use the standard kit to test accuracy of the standard tube into the DensiCHEKTM Plus prior to first use and on a monthly basis.

Important instructions for use

- Confirm that the DensiCHEKTM Plus instrument is set to the GLASS tube setting.
- Select the 0.0 McF standards and clean the outside surface with a lens tissue.
- Gently invert the 0.0 McF standard five to six times to ensure it is homogenous.
- Ensure the instrument is on and insert the 0.0 McF standards into the instrument and press the Zero/Scroll key.
- Slowly rotate the standard one full rotation. The instrument will display a series of dashes followed by 0.00.
- Select the desired standard (0.5, 2.0, and 3.0) and clean outside surface with lens tissue.
- Gently invert the standard five to six times to ensure it is homogenous.
- Insert the standard into the instrument and slowly rotate the standard one full rotation until a numerical value is displayed.
- Check the displayed McFarland value is within the acceptable range.
- If any standards are outside the acceptable range repeat above steps. If it is still out of the range contact with vendors.

Reference range:

Standard	Acceptable Range
0.5 McF	0.44 - 0.56
2.0 McF	1.85 – 2.15
3.0 McF	2.79 – 3.21

Safety precautions

- For in vitro diagnostic use only.
- Suspension not within the appropriate range on VITEK 2 Densichek may compromise
- card performance
- Do not use card after the expiration date shown on the package liner
- Allow the card to come to room temperature before use.

Only pure isolates are used for sensitivity testing. Mixed cultures used for testing give erroneous results. In case, inoculum has a mixed growth as identified by 'unidentified' flag by Vitek, the isolate is subcultured and the test is repeated.

Unusual results (Recheck result and isolate purity before reporting)

- *Klebsiella* sensitive to ampicillin
- *Staphylococcus* resistant to vancomycin
- MRSA should not be reported as sensitive to any beta lactam antibiotics.

5.1.4 Agar dilution method

5.1.4.1 Media

Prepare Mueller-Hinton agar plates containing the correct amount of the antibiotic. For this use sterile McCartney bottles (or appropriate tubes) with 20 ml of sterile agar media cooled to 48-50 ºC in a water bath. Add 256 µl, 128 µl, 56 µl and 28 µl of antibiotic stock 1 (10,000 µg/ml); 160 µl, 80 µl, and 40 µl of stock 2 (1,000 µg/ml); and 200 µl, 100 µl, and 50 µl of stock 3 (100 µg/ml) to get media containing antibiotic concentration 128 µg/ml to 0.25 µg/ml respectively. Mix well the content of McCartney bottles and pour into petri plates (90 mm). Allow the plates to solidify and dry. Depth of media should be 3-4 mm. Use without sterility check as antibiotics degrade if plates are incubated. Make a checkerboard on the back of the plates to identify spots for various strains.

Note: MHA should contain 20-25 mg/L of Ca^{++} ions and 10-12.5 mg/L of Mg^{++} ions. At least two plates with no antimicrobial should be prepared as growth control and purity plates.

5.1.4.2 Inoculum

To get pure culture of target organism, suspend either 3-5 colonies in normal saline or use the growth method as described previously to get an inoculum of 0.5 McFarland (1.5×10^8 cfu/ml). Make 1 in 100 dilution of the suspension by adding 0.1 ml growth into 9.9 ml

normal saline to obtain ~10^6 cfu/ml. Carefully spot the 1µl on to the agar plate to get the final inoculum of 10^4 cfu. Spot 10^3 cfu for sulphonamides.

5.1.4.3 Incubation: 35-37 ºC
Controls: Use one negative control and positive control spot.

5.1.4.4 Period of incubation: 16-20 hours except for oxacillin for *Staphylococcus,* and vancomycin for *Enterococcus* where it should be 24 hours.

5.1.4.5 Reading: MIC is the lowest concentration of antibiotic at which there is no visible growth. Ignore film or one or two colonies.

5.1.5 MIC strips or E-Test method

5.1.5.1 Inoculum

Remove the E-test package from the freezer (-20 ºC) at least 30 minutes before use. With a loop, touch the top of 3 or 4 individual colonies and transfer to a tube of saline. Emulsify the inoculum on the inside of the tube to avoid lumps. Compare and adjust the turbidity to match the 0.5 McFarland standard.

5.1.5.2 Inoculation

Use Mueller-Hinton agar plates and ensure the agar surface is dry, but not overly dry. Swab the plate within 15 minutes of preparing the inoculum. Dip a sterile cotton swab into the inoculum, pull out slightly and rotate the swab several times against the inside of the tube above the fluid level to remove excess liquid. Streak the swab over the entire surface of the agar plate. Rotate the plate approximately 60 ºC then repeat streaking motion. Rotate 60 ºC again and repeat streaking. Complete inoculation by running the swab around the rim of the agar. Leave the lid of the plate ajar for 5 minutes (no more than 15 minutes) to allow absorption of any excess moisture before applying strips.

5.1.5.3 E-test strip application

Apply strips to agar surface using forceps (or E-test applicator if available). Place the strip with the ‘E end’ at the center of the plate and with the scale visible. Position one (seldom two) strip(s) onto a 90 mm plate or 4-6 strips onto a 150 mm plate. Do not remove a strip once it has touched the agar.

5.1.5.4 Incubation: 37 ºC for 18 hours in ambient air.

5.1.5.5 Results: Read MIC at the point where ellipse intersects the scale. If a MIC value is seen between two dilutions (2 fold), always round up to the highest value. Remember to read the MIC value at complete inhibition of all growth including isolated colonies. If the intersect differs on either side of the strip, read the greater value as the MIC. However, sulfonamide and trimethoprim should be read at 80% of growth. Ignore swarming of *Proteus* spp., and any growth at the edge of the strip.

AST and MIC Breakpoints

CHAPTER 6

AST AND MIC BREAKPOINTS

Antimicrobial agents recommended for testing as per CLSI guidelines -VET08[19]

	Swine	Cattle [a]	Bovine-Mastitis [b]	Poultry [c]	Horses	Dogs* & Cats[$]
elopmentAble may be retained for sake of future dGroup A – Veterinary Specific Breakpoints Primary Test and Report	Ceftiofur[d]	Ceftiofur [d]	Ceftiofur [d]	Enrofloxacin[d]	Amikacin Gentamicin[m]	Amikacin* Gentamicin*[m]
						Amoxicillin-Clavulanate Piperacillin-Tazonactam*
		Spectinomycin	Pirlimycin		Cefazolin[m] Ceftiofur	Cefovecin Cefpodoxime* Cephalexin*[m] Cephalothin*[m] Cefazolin*[m]
	Tildipirosin Tilmicosin Tulathromycin	Gamithromycin Tildipirosin Tilmicosin Tulathromycin	Penicillin - Novobiocin			Clindamycin*
	Ampicillin [f,m] Penicillin G [m]	Ampicillin [f] Penicillin G [m]			Ampicillin[f,m] Penicillin G [m]	Ampicillin[$f] Ampicillin*[f,m]
	Florfenicol	Florfenicol				
	Tiamulin	Danofloxacin [d]				
	Enrofloxacin [d]	Enrofloxacin [d]			Enrofloxacin[m]	Difloxacin* Enrofloxacin Marbofloxacin Orbifloxacin Pradifloxacin
	Tetracycline [i]	Tetracycline [i]			Doxycycline[m] Minocycline[m]	Doxycycline* Minocycline* Tetracycline*[i]

	Swine	Cattle[a]	Bovine-Mastitis [b]	Poultry[c]	Horses	Dogs* & Cats[$]
Group B – CLSI-Approved Human Breakpoints Primary Test, Selectively Report	Gentamicin	Sulfonamides	Cefoperazone [d] Cephalothin[g]	Spectinomycin	Sulfonamides Trimethoprim-sulfamethoxazole[j]	Amikacin[$] Gentamicin[$]
	Clindamycin[e]	Erythromycin	Erythromycin	Gentamicin	Erythromycin	Cephalothin[$] Cephalexin[$] Cefazolin[$]
	Sulfonamides		Ampicillin[f] Oxacillin[h] Penicillin	Sulfonamides Trimethoprim-sulfamethoxazole[j]	Chloramphenicol[k]	Sulfonamides Trimethoprim-Sulfamethoxazole[j]
	Erythromycin		Tetracycline[i]	Erythromycin	Tetacycline [i]	Clindamycin[$]
				Penicillin (Turkeys only)		Erythromycin
				Tetracycline[i]		Oxacillin[h] Penicillin
						Chloramphenicol[k]
						Doxycycline[$] Tetracycline[$i]

Group C – No Veterinary Species specific or Human-specific Breakpoints Primary Test, Selectively Report	Swine	Cattle[a]	Bovine-Mastitis [b]	Poultry[c]	Horses	Dogs* & Cats[$]
	Apramycin Spectinomycin	Cefquinome[d]	Kanamycin-Cephalexin[d]	Spectinomycin	Cefquinome	Specitomycin
	Cefquinome[d]	Tylosin	Cefquinome[d]	Ceftiofur (chickens only) [d]		Ceftiofur*
	Tylosin			Clindamycin[c]		

Group D Supplemental Selectively Test, Selectively Report	Swine	Cattle[a]	Bovine-Mastitis [b]	Poultry[c]	Horses	Dogs* & Cats[$]
	Amikacin	Amikacin[l] Gentamicin[l]		Tylvalosin[d]	Rifampin	Tobramycin
	Trimethoprim-Sulfamethoxazole[j]	Trimethoprim-Sulfamethoxazole [j]			Imipenem	Imipenem
	Tylvalosin				Vancomycin	Vancomycin
	Valnemulin[d]				Oxacillin	Nitrofuratoin (Urine only)

a. Does not include goats or sheep.
b. Only compounds approved for use in lactating dairy cattle by intramammary infusion are listed.
c. Includes chickens and turkeys.
d. This drug is not approved in the United States and Canada, or this drug is prohibited from certain extra-label uses in the United States, but may be approved in other countries. (Check country and local regulations; also, see NOTE 5 for website references).
e. Clindamycin is used for susceptibility to lincomycin. In poultry, lincomycin is for chickens only.
f. Ampicillin is the class representative for aminopenicillins and should be tested.
g. The results of cephalothin susceptibility tests are used to predict susceptibility to cephapirin for bovine mastitis.
h. The results of oxacillin susceptibility tests are used to predict susceptibility to cloxacillin. Oxacillin-resistant staphylococci should be reported as resistant to all β-lactams.
i. The results of tetracycline susceptibility tests are used to predict susceptibility for chlortetracycline and oxytetracycline. Alternatively, chlortetracycline or oxytetracycline can be used for primary testing.
j. The results of the trimethoprim-sulfamethoxazole can be used to predict the susceptibility of potentiated sulfonamides containing trimethoprim. There are no data on the ability of trimethoprim-sulfamethoxazole results to predict suceptibility to ormetoprim-sulfadimethoxine combinations. Trimethoprim-sulfadiazine and ormetoprim- sulfadimethoxine are approved in dogs.
k. Chloramphenicol is banned from use in food animals in the United States and many other countries; therefore, it must not be reported with any food producing animal species.
l. Due to extended residue times, extra-label use of aminoglycosides in cattle should be avoided.
m. Breakpoints were established based on the extra-lable use of this drug.

6.1 Breakpoints for both clinical specimen and surveillance specimen[19].

6.1.1 Clinical specimen

Table 6.1: Zone diameters and MIC BPs for *E. coli* isolated from dog.

Animal	AMA	Body site	Disk content (µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Dog	Amikacin		-	-	-	-	≤4	8	≥16
	Amoxicillin-clavulanate	SST	-	-	-	-	≤0.25/ 0.12	0.5/0.25	≥1/0.5
	Amoxicillin-clavulanate	UT	-	≥18	-	-	≤8/4	-	-
	Ampicillin	SST	-	-	-	-	≤0.25	0.5	≥1
	Ampicillin	UT	-	-	-	-	≤8	-	-
	Cefazolin	SST	-	-	-	-	≤2	4	≥8
	Cefazolin	UT	-	-	-	-	≤16	-	≥32
	Ceftiofur*		30	≥21	18-20	≤17	≤2	4	≥8
	Enrofloxacin	SST, RT, UT	5	≥23	17-22	≤16	≤0.5	1-2	≥4
	Gentamicin	-	10	≥16	13-15	≤12	≤2	4	≥8
	Piperacillin-tazobactam	SST, UT	-	-	-	-	≤8/4	16/4	≥32/4
Human	Ceftriaxone		30	≥23	20-22	≤19	≤1	2	≥4
	Ciprofloxacin		5	≥26	21-25	≤21	≤0.25	0.5	≥1
	Imipenem		10	≥23	20-22	≤19	≤1	2	≥4
	Meropenem		10	≥23	20-22	≤19	≤1	2	≥4
	Nitrofurantoin		300	≥17	15-16	≤14	≤32	64	≥128
	Trimethoprim-sulfamethoxazole		1.25/ 23.7	≥16	11-15	≤10	≤2/38	-	≥4/76

AMA: antimicrobial agent; BPs: breakpoints; SST: skin and soft tissue; UT: urinary tract; RT: respiratory

Notes:

(i) *BPs for indicated organisms isolated from the Dog is not available, so BPs from other animal species (cattle/swine) can be utilized.

(ii) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

Table 6.2: Zone diameters and MIC BPs for non-*Escherichia coli* Enterobacteriaceae isolated from dog.

Animal	AMA	Body site	Disk content (µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Dog	Cefazolin*	UT	-	-	-	-	≤16	-	≥32
	Ceftiofur[@]		30	≥21	18-20	≤17	≤2	4	≥8
	Enrofloxacin	SST, RT, UT	5	≥23	17-22	≤16	≤0.5	1-2	≥4
	Gentamicin	-	10	≥16	13-15	≤12	≤2	4	≥8
	Piperacillin-tazobactam	SST, UT	-	-	-	-	≤8/4	16/4	≥32/4
Human	Amikacin		30	≥17	15-16	≤14	≤16	32	≥64
	Amoxicillin-clavulanate		20/10	≥18	14-17	≤13	≤8/4	16/8	≥32/16
	Ampicillin		10	≥17	14-16	≤13	≤8	16	≥32
	Cefazolin		30	≥23	20-22	≤19	≤2	4	≥8
	Cefazolin	UT	30	≥15	-	≤14	≤16	-	≥32
	Ceftriaxone		30	≥23	20-22	≤19	≤1	2	≥4
	Chloramphenicol		30	≥18	13-17	≤12	≤8	16	≥32
	Ciprofloxacin		5	≥26	21-25	≤21	≤0.25	0.5	≥1
	Ciprofloxacin[#]		5	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
	Imipenem		10	≥23	20-22	≤19	≤1	2	≥4
	Meropenem		10	≥23	20-22	≤19	≤1	2	≥4
	Nitrofurantoin		300	≥17	15-16	≤14	≤32	64	≥128
	Trimethoprim-sulfamethoxazole		1.25/23.7	≥16	11-15	≤10	≤2/38	-	≥4/76

AMA: antimicrobial agent; BPs: breakpoints; SST: skin and soft tissue; UT: urinary tract; RT: respiratory

Notes:

(i) *: Specifically for *K. pneumoniae*, #: Specifically for *Salmonella* spp.

(ii) [@]BPs for indicated organisms isolated from the Dog is not available, so BPs from other animal species (cattle/swine) can be utilized.

(iii) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

(iv) For faecal isolates of *Salmonella*, test only ampicillin, fluoroquinolone and trimethoprim-sulfamethoxazole. In addition, for extraintestinal *Salmonella*, third generation cephalosporin and chloramphenicol may be tested.

Table 6.3: Zone diameters and MIC BPs for Enterobacteriaceae isolated from cattle.

Animal	AMA	Body site	Disk content (µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Cattle	Ampicillin*	Metritis	-	-	-	-	≤0.25	0.5	≥1
	Ceftiofur*	Mastitis	30	≥21	18-20	≤17	≤2	4	≥8
	Enrofloxacin[#]		5	≥23	17-22	≤16	≤0.5	1-2	≥4
Human	Amikacin		30	≥17	15-16	≤14	≤16	32	≥64
	Ampicillin		10	≥17	14-16	≤13	≤8	16	≥32
	Amoxicillin-clavulanate		20/10	≥18	14-17	≤13	≤8/4	16/8	≥32/16
	Cefazolin		30	≥23	20-22	≤19	≤2	4	≥8
	Cefazolin	UT	30	≥15	-	≤14	≤16	-	≥32
	Ceftriaxone		30	≥23	20-22	≤19	≤1	2	≥4
	Chloramphenicol		30	≥18	13-17	≤12	≤8	16	≥32
	Ciprofloxacin		5	≥26	21-25	≤21	≤0.25	0.5	≥1
	Ciprofloxacin[#]		5	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
	Gentamicin		10	≥15	13-14	≤12	≤4	8	≥16
	Imipenem		10	≥23	20-22	≤19	≤1	2	≥4
	Meropenem		10	≥23	20-22	≤19	≤1	2	≥4
	Nitrofurantoin		300	≥17	15-16	≤14	≤32	64	≥128
	Piperacillin-tazobactam		100/10	≥21	18-20	≤17	≤16/4	32/4-64/4	≥128/4
	Tetracycline		30	≥15	12-14	≤11	≤4	8	≥16
	Trimethoprim-sulfamethoxazole		1.25/23.7	≥16	11-15	≤10	≤2/38	-	≥4/76

AMA: antimicrobial agent; BPs: breakpoints; UT: urinary tract

Notes:

(i) *: Specifically for *E. coli.*

(ii) *BPs for indicated organisms isolated from the cattle is not available, so BPs from other animal species (Dog/ cat) can be utilized.

(iii) [#]: Specifically for *Salmonella* spp.

(iv) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

(v) For faecal isolates of *Salmonella*, test only ampicillin, fluoroquinolone and trimethoprim-sulfamethoxazole. In addition, for extra-intestinal *Salmonella*, third generation cephalosporin and chloramphenicol may be tested.

Table 6.4: Zone diameters and MIC BPs for Enterobacteriaceae isolated from swine.

Animal	AMA	Body site	Disk content (µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Swine	Ceftiofur[@]	RT	30	≥21	18-20	≤17	≤2	4	≥8
	Enrofloxacin*		5	≥23	17-22	≤16	≤0.5	1-2	≥4
Human	Amikacin		30	≥17	15-16	≤14	≤16	32	≥64
	Ampicillin		10	≥17	14-16	≤13	≤8	16	≥32
	Cefazolin		30	≥23	20-22	≤19	≤2	4	≥8
	Cefazolin	UT	30	≥15	-	≤14	≤16	-	≥32
	Ceftriaxone		30	≥23	20-22	≤19	≤1	2	≥4
	Ciprofloxacin		5	≥26	21-25	≤21	≤0.25	0.5	≥1
	Ciprofloxacin[#]		5	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
	Imipenem		10	≥23	20-22	≤19	≤1	2	≥4
	Meropenem		10	≥23	20-22	≤19	≤1	2	≥4
	Nitrofurantoin		300	≥17	15-16	≤14	≤32	64	≥128
	Piperacillin-tazobactam		100/10	≥21	18-20	≤17	≤16/4	32/4-64/4	≥128/4
	Trimethoprim-sulfamethoxazole		1.25/23.7	≥16	11-15	≤10	≤2/38	-	≥4/76

AMA: antimicrobial agent; BPs: breakpoints; UT: urinary tract; RT: respiratory

Notes:

(i) [@]: Specifically for *S. choleraesuis.*

(ii) *BPs for indicated organisms isolated from the cattle is not available, so BPs from other animal species (Dog/ cat) can be utilized.

(iii)[#]: Specifically for *Salmonella* spp.

(iv) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

(v) For faecal isolates of *Salmonella,* test only ampicillin, fluoroquinolone and trimethoprim-sulfamethoxazole. In addition, for extraintestinal *Salmonella,* third generation cephalosporin and chloramphenicol may be tested.

Table 6.5: Zone diameters and MIC BPs for *Staphylococcus* isolated from dog.

Animal	AMA	Body site	Disk content (µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Dog	Amikacin		-	-	-	-	≤4	8	≥16
	Amoxicillin-clavulanate	SST	-	-	-	-	≤0.25/ 0.12	0.5/0. 25	≥1/0. 5
	Amoxicillin-clavulanate	UT	-	-	-	-	≤8/4	-	-
	Ceftiofur								
	Clindamycin	SST	2	≥21	15-20	≤14	≤0.5	1-2	≥4
	Enrofloxacin	SST, UT, RT	5	≥23	17-22	≤16	≤0.5	1-2	≥4
	Tetracycline	SST	30	≥23	18-22	≤17	≤0.25	0.5	≥1
Human	Cefoxitin*&		30	≥22	-	≤21	≤4	-	≥8
	Cefoxitin**		30	≥25	-	≤24	-	-	-
	Cefoxitin****		30	≥25	-	≤24	-	-	-
	Erythromycin		15	≥23	14-22	≤13	≤0.5	1-4	≥8
	Gentamicin		10	≥15	13-14	≤12	≤4	8	≥16
	Oxacillin*&		-	-	-	-	≤2	-	≥4
	Oxacillin**		1	≥18	-	≤17	≤0.25	-	≥0.5
	Oxacillin***		1	≥18	-	≤17	≤0.25	-	≥0.5
	Oxacillin****		-	-	-	-	≤0.25	-	≥0.5
	Trimethoprim-sulfamethoxazole		1.25/ 23.75	≥16	11-15	≤10	≤2/38	-	≥4/76
	Vancomycin*		-	-	-	-	≤2	4-8	≥16
	Vancomycin@		-	-	-	-	≤4	8-16	≥32

AMA: antimicrobial agent; BPs: breakpoints; SST: skin and soft tissue; UT: urinary tract; RT: respiratory

Notes:

(i) *: Specifically for *S. aureus*

(ii) **: *Staphylococcus epidermidis*

(iii) ***: Specifically for *S. pseudointermedius* and *S.schleiferi*

(iv) ****: Other Staphylococcus spp. excluding *S. aureus, S. lugdunensis, S. epidermidis, S. pseudointermedius* and *S.schleiferi*

(v) @: All CoNS

(vi) &: Specifically for *S. lugdunensis.*

(vii) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

(viii) Cefoxitin (oxacillin) susceptible staphylococci are susceptible to all penicillinase stable penicillins. Cefoxitin (oxacillin) resistant staphylococci are resistant to all β-lactam antibiotics except MRSA susceptible newer penicillin's (no advantage of testing ampicillin and amoxicillin-clavulanate)

Table 6.6: Zone diameters and MIC BPs for *Staphylococcus* isolated from cattle.

Animal	AMA	Body site	Disk content(µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Cattle	Ceftiofur*	udder	30	≥21	18-20	≤17	≤2	4	≥8
Human	Cefoxitin*&		30	≥22	-	≤21	≤4	-	≥8
	Cefoxitin**		30	≥25	-	≤24	-	-	-
	Cefoxitin****		30	≥25	-	≤24	-	-	-
	Clindamycin		2	≥21	15-20	≤14	≤0.5	1-2	≥4
	Erythromycin		15	≥23	14-22	≤13	≤0.5	1-4	≥8
	Gentamicin		10	≥15	13-14	≤12	≤4	8	≥16
	Oxacillin*&		-	-	-	-	≤2	-	≥4
	Oxacillin**		1	≥18	-	≤17	≤0.25	-	≥0.5
	Oxacillin***		1	≥18	-	≤17	≤0.25	-	≥0.5
	Oxacillin****		-	-	-	-	≤0.25	-	≥0.5
	Tetracycline		30	≥19	15-18	≤14	≤4	8	≥16
	Trimethoprim-sulfamethoxazole		1.25/ 23.75	≥16	11-15	≤10	≤2/38	-	≥4/76
	Vancomycin*		-	-	-	-	≤2	4-8	≥16
	Vancomycin@		-	-	-	-	≤4	8-16	≥32

AMA: antimicrobial agent; BPs: breakpoints

Notes:

(i) *: Specifically for *S. aureus*

(ii) **: *Staphylococcus epidermidis*

(iii) ***: Specifically for *S. pseudointermedius* and *S.schleiferi*

(iv) ****: Other Staphylococcus spp. excluding *S. aureus, S. lugdunensis, S. epidermidis, S. pseudointermedius* and *S.schleiferi*

(v) @: All CoNS

(vi) &: Specifically for *S. lugdunensis*

(vii) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

(viii) Cefoxitin (oxacillin) susceptible staphylococci are susceptible to all penicillinase stable penicillins. Cefoxitin (oxacillin) resistant staphylococci are resistant to all β-lactam antibiotics except MRSA susceptible newer penicillins (no advantage of testing ampicillin and amoxicillin-clavulanate).

Table 6.7: Zone diameters and MIC BPs for *Staphylococcus* isolated from swine.

Animal	AMA	Body site	Disk Content(µg)	Zone size (mm)			MIC (µg/ml)		
				S	I	R	S	I	R
Human	Cefoxitin*&		30	≥22	-	≤21	≤4	-	≥8
	Cefoxitin**		30	≥25	-	≤24	-	-	-
	Cefoxitin****		30	≥25	-	≤24	-	-	-
	Clindamycin		2	≥21	15-20	≤14	≤0.5	1-2	≥4
	Erythromycin		15	≥23	14-22	≤13	≤0.5	1-4	≥8
	Gentamicin		10	≥15	13-14	≤12	≤4	8	≥16
	Oxacillin*&		-	-	-	-	≤2	-	≥4
	Oxacillin**		1	≥18	-	≤17	≤0.25	-	≥0.5
	Oxacillin***		1	≥18	-	≤17	≤0.25	-	≥0.5
	Oxacillin****		-	-	-	-	≤0.25	-	≥0.5
	Tetracycline		30	≥19	15-18	≤14	≤4	8	≥16
	Trimethoprim-sulfamethoxazole		1.25/ 23.75	≥16	11-15	≤10	≤2/38	-	≥4/76
	Vancomycin*		-	-	-	-	≤2	4-8	≥16
	Vancomycin@		-	-	-	-	≤4	8-16	≥32

AMA: antimicrobial agent; BPs: breakpoints

Notes:

(i) *: Specifically for *S. aureus*

(ii) **: *Staphylococcus epidermidis*

(iii)***: Specifically for *S. pseudointermedius* and *S.schleiferi*

(iv)****: Other Staphylococcus spp. excluding *S. aureus, S. lugdunensis, S. epidermidis, S. pseudointermedius* and *S.schleiferi*

(v) @: All CoNS

(vi)&: Specifically for *S. lugdunensis*

(vii) If veterinary specific BPs for indicated organisms isolated from the designated animal species (with defined disease) for the specific antimicrobial agents are not available, then BPs from human isolates can be utilized.

(viii)Cefoxitin (oxacillin) susceptible staphylococci are susceptible to applies to all penicillinase stable penicillins. Cefoxitin (oxacillin) resistant staphylococci are resistant to all β-lactam antibiotics except MRSA susceptible newer penicillins (no advantage of testing ampicillin and amoxicillin-clavulanate).

6.1.2 Surveillance specimen

Table 6.8: Zone diameters and MIC BPs for non- *Salmonella* Enterobacteriaceae.

AMA	Disk content (µg)	Zone size mm			MIC µg/ml		
		S	I	R	S	I	R
Amikacin	30	≥17	15-16	≤14	≤16	32	≥64
Amoxicillin-clavulanate	20/10	≥18	14-17	≤13	≤8/4	16/8	≥32/16
Ampicillin	10	≥17	15-16	≤14	≤8	16	≥32
Cefazolin	30	≥23	20-22	≤19	≤2	4	≥8
Ceftiofur *	30	≥21	18-20	≤17	≤2	4	≥8
Cefotaxime	30	≥26	23-25	≤22	≤1	2	≥4
Ceftazidime	30	≥ 21	18-20	≤ 17	≤ 4	8	≥ 16
Chloramphenicol	30	≥18	13-17	≤12	≤8	16	≥32
Ciprofloxacin	5	≥26	22-25	≤21	≤0.25	0.5	≥1
Colistin [#]	-	-	-	-	≤2	-	≥4
Gentamicin	10	≥15	13-14	≤12	≤4	8	≥16
Imipenem	10	≥23	20-22	≤19	≤1	2	≥4
Meropenem	10	≥23	20-22	≤19	≤1	2	≥4
Nalidixic acid	30	≥19	14-18	≤13	≤16	-	≥32
Tetracycline	30	≥15	12-14	≤11	≤4	8	≥16
Trimethoprim-sulfamethoxazole	1.25/23.7	≥16	11-15	≤10	≤2/38	-	≥4/76

AMA: antimicrobial agent; BPs: breakpoints

Notes:

(i) *: CLSI VET -01-S-Ed-3

(ii) [#] Colistin to be tested only by MIC test. EUCAST guidelines, 2015 give cut offs only for colistin.

Table 6.9: Zone diameters and MIC BPs for *Salmonella* spp

AMA	Disk Content (µg)	Zone size mm			MIC µg/ml		
		S	I	R	S	I	R
Ampicillin	10	≥17	14-16	≤13	≤8	16	≥32
Azithromycin	15	≥13	-	≤12	≤16	-	≥32
Ceftriaxone	30	≥23	20-22	≤19	≤1	2	≥4
Chloramphenicol	30	≥18	13-17	≤12	≤8	16	≥32
Ciprofloxacin	5	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
Nalidixic acid	30	≥19	14-18	≤13	≤16	-	≥32
Tetracycline	30	≥15	12-14	≤11	≤4	8	≥16
Trimethoprim-sulfamethoxazole	1.25/23.7	≥16	11-15	≤10	≤2/38	-	≥4/76

AMA: antimicrobial agent; BPs: breakpoints

Table 6.10: Zone diameters and MIC BPs for *Staphylococcus* spp.

AMA	Disk Content (µg)	Zone size (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Cefoxitin*&	30	≥22	-	≤21	≤4	-	≥8
Cefoxitin**	30	≥25	-	≤24	-	-	-
Cefoxitin****	30	≥25	-	≤24	-	-	-
Chloramphenicol	30	≥18	13-17	≤12	≤8	16	≥32
Ciprofloxacin	5	≥21	16-20	≤15	≤1	2	≥4
Clindamycin	2	≥21	15-20	≤14	≤0.5	1-2	≥4
Erythromycin	15	≥23	14-22	≤13	≤0.5	1-4	≥8
Gentamicin	10	≥15	13-14	≤12	≤4	8	≥16
Linezolid	30	≥21	-	≤20	≤4	-	≥8
Oxacillin*&	-	-	-	-	≤2	-	≥4
Oxacillin**	1	≥18	-	≤17	≤0.25	-	≥0.5
Oxacillin***	1	≥18	-	≤17	≤0.25	-	≥0.5
Oxacillin****	-	-	-	-	≤0.25	-	≥0.5
Penicillin	10 units	≥29	-	≤28	≤0.12	-	≥0.25
Tetracycline	30	≥19	15-18	≤14	≤4	8	≥16
Trimethoprim-sulfamethoxazole	1.25/ 23.75	≥16	11-15	≤10	≤2/38	-	≥4/76
Vancomycin*	-	-	-	-	≤2	4-8	≥16
Vancomycin@	-	-	-	-	≤4	8-16	≥32

AMA: antimicrobial agent; BPs: breakpoints

(i) *: Specifically for *Staphylococcus aureus.*

(ii) **: *Staphylococcus epidermidis*

(iii) ***: Specifically for *S. pseudointermedius* and *S.schleiferi*

(iv) ****: Other Staphylococcus spp. excluding *S. aureus, S. lugdunensis, S. epidermidis, S. pseudointermedius* and *S.schleiferi*

(v) @: All CoNS (vi) &: Specifically for *S. lugdunensis.*

Table 6.11: Zone diameters and MIC BPs for *Enterococcus* spp.

AMA (antimicrobial agent)	Disk Content (µg)	Zone size (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Ampicillin	10	≥17	-	≤16	≤8	-	≥16
Chloramphenicol	30	≥18	13-17	≤12	≤8	16	≥32
Ciprofloxacin	5	≥21	16-20	≤15	≤1	2	≥4
Doxycycline	30	≥16	13-15	≤12	≤4	8	≥16
Erythromycin	15	≥23	14-22	≤13	≤0.5	1-4	≥8
Gentamicin High Level	120	≥10	7-9	≤6	≤500	-	≥1000
Linezolid	30	≥23	21-22	≤20	≤2	4	≥8
Minocycline	30	≥19	15-18	≤14	≤4	8	≥16
Nitrofurantoin	300	≥17	15-16	≤14	≤32	64	≥128
Tetracycline	30	≥19	15-18	≤14	≤4	8	≥16
Vancomycin	30	≥17	15-16	≤14	≤4	8-16	≥32

Quality Control (QC)

CHAPTER 7

QUALITY CONTROL (QC) IN PERFORMING SUSCEPTIBILITY TESTS

7.1 Quality control responsibilities of a laboratory (CLSI- VET method[16)].

- Storage under the environmental conditions as recommended by the manufacturer
- Proficiency of personnel performing tests
- Adherence to established procedure (e.g. Inoculum preparation, incubation conditions, interpretation of end points)
- Batch or lot control (e.g. media, disks etc.)
- Maintenance of a QC test logs

Table 7.1: Reference strains for quality control

	Testing purpose	**ATCC® Strain**	
Enterobacteriaceae	Disk diffusion	*E. coli*	25922
	For β-lactam/ β-lactamase inhibitor combination	*E. coli*	35218
	For carbapenems	*P. aeruginosa*	27853
Staphylococcus spp.	Disk diffusion	*S. aureus*	25923
	MIC	*S. aureus*	29213
	For β-lactam/ β-lactamase inhibitor combination	*E. coli*	35218
Enterococcus spp.	Disk diffusion	*S. aureus*	25923
	Dilution method	*E. faecalis*	29212
CoNS	β-lactamase (positive)	*S. aureus*	29213
	(negative)	*S. aureus*	25923
ESBL	Disk diffusion	*K. pneumoniae*	700603
		or	
		E. coli	25922
Clindamycin	Routine QC disk Diffusion	*S. aureus*	25923
	Broth microdilution	*S. aureus*	29213
Oxacillin R	Susceptible (S)	*S. aureus*	29213
	Resistance (R)	*S. aureus*	43300
Reduced S to Vancomycin	vancomycin -R	*E. faecalis*	51299
	vancomycin - S	*E. faecalis*	29212

Note: It might be necessary to check the MHA when sulfonamides, trimethoprim, or trimethoprim-sulfamethoxazole are tested routinely. It is especially important for some

veterinary pathogens such as *S. hyicus*. To evaluate a new lot of MHA, *E. faecalis* ATCC® 29212 or *E. faecalis* ATCC® 33186 alternatively, may be tested with trimethoprim-sulfamethoxazole disks. Satisfactory media will provide essentially clear, distinct zones of inhibition of ≥20 mm, while unsatisfactory media will produce no zone of inhibition, growth within the zone, or a zone ≤ 20 mm.

7.2 Storing and testing QC strains

Preserve the strains in BHI broth with 10% glycerol at -20ºC or -70ºC or liquid nitrogen for prolonged storage, else lyophilize the strains. Working cultures should be stored on trypticase soya agar (TSA) or other nonselective media (MHA), blood/chocolate agar plates at 2-8 ºC. Fresh working culture needs to be prepared if test values fall outside the acceptable range. These strains should be tested by the standard procedures mentioned in section 4.2. Zone sizes should be compared with that mentioned in the table for control strains. Before testing, subculture strains to obtain isolated colonies. Freeze dried or frozen cultures should be sub-cultured twice prior to testing. If an unexplained result suggests a change in the organism's inherent susceptibility, a new culture of the control strain should be obtained.

7.3 Frequency of testing

Daily quality control testing is advised at the beginning for at least 20-30 days. When no more than 1 out of 20, or 3 out of 30 consecutive tests turn out to be outside acceptable limit, performance should be considered satisfactory to proceed to weekly testing. If not, corrective action should be taken. Daily testing should also be required when manufacturer of agar media are changed, or when upgrading results reading from manual to automated system. Along with weekly testing, QC should be performed whenever reagent component of the test is changed. If any weekly test result is out of range, corrective action should be taken.

Note: QC of tests with the negative control (susceptible strain) is recommended each testing day if the test is not performed routinely (i.e. at least once a week). Frequent QC testing (more than once a week) may be needed for rapidly degrading drugs.

7.4 Quality control of media

Mueller Hinton agar or various other broth and agar media not containing antimicrobials should always be kept overnight at 37ºC for sterility checking prior to inoculation. Each batch or new lot of media, lot of disks, macrodilution tubes, microdilution trays, or agar dilution plates should be checked with reference ATCC® strains. At least one uninoculated tube, broth microdilution tray, or agar plate from each batch should be incubated overnight to confirm the medium's sterility. Zone sizes and MICs obtained must be within acceptable CLSI limits. If not, corrective actions are required. The batch should be rejected in case of unsuccessful corrective action. Acceptable cation content should be suspected if QC errors are encountered with antimicrobial agents that are more likely to get affected by cation

variations (e.g. aminoglycosides). Performance tests should be done with each lot of Mueller-Hinton agar and broth for checking variations in divalent cations using gentamicin and *P. aeruginosa* ATCC® 27853. If the MIC results are out of range on the low end, broth may need additional cation supplementation.

7.5 Corrective action for out-of-range results

Out-of-range QC results could occur due to random (due to chance), identifiable (e.g. QC strain, testing supplies and process, equipment etc.) or system related errors. The random and identifiable errors can usually be resolved by a single repeat of the QC test. However, test system related errors usually do not correct when the QC test is repeated and may indicate a serious problem which must be investigated. It may be necessary to use an alternative test method until the problem is resolved.

7.6 Record maintenance

Records to maintain must include date of testing, organism tested, ATCC® strain, test method, media used, lot numbers, expiration dates, date of use of all materials and reagents, antibiotic used, control limits, test results, and remarks in performing susceptibility tests.

Special Tests

CHAPTER 8

SPECIAL ANTIMICROBIAL SUSCEPTIBILITY TESTS (PHENOTYPIC)

8.1 Detection of Extended Spectrum β-Lactamase (ESBL) producing Enterobacteriaceae

Table 8.1a: Screening and confirmatory tests for ESBLs in *Klebsiella pneumoniae, K. oxytoca,* and *Escherichia coli* (zone diameter)

Method	**Initial screen test**	**Phenotypic confirmatory test**
Medium	**Mueller-Hinton agar**	**Mueller-Hinton agar**
Antimicrobial Disk content	For *K. pneumoniae, K. oxytoca, E. coli:* Cefpodoxime 10 µg or Ceftazidime 30 µg or Aztreonam 30 µg or Cefotaxime 30 µg or Ceftriaxone 30 µg (Use of more than one antimicrobial agent for screening improves the sensitivity of detection).	Ceftazidime 30 µg Ceftazidime-clavulanic acid 30/10 µg And Cefotaxime 30 µg Cefotaxime-clavulanic acid 30/10 µg (Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid).
Inoculum Incubation length and conditions	Standard disk diffusion recommendations as described above	Standard disk diffusion recommendations as described above
Results	Cefpodoxime ≤ 17 mm Ceftazidime ≤ 22 mm Aztreonam ≤ 27 mm Cefotaxime ≤ 27 mm Ceftriaxone ≤ 25 mm Zones above may indicate ESBL production	A ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone = ESBL (e.g. ceftazidime zone = 16; ceftazidime-clavulanic acid zone=21)
QC recommendation	When testing ESBL-screening antimicrobial agents, *K. pneumoniae* ATCC® 700603 should be used for quality assessment (e.g. training, competency, or test evaluation). Either strain, *K. pneumoniae* ATCC® 700603 or *E. coli* ATCC® 25922 should be used for routine QC (e.g. weekly or daily). ***E. coli* ®25922 / *K. pneumoniae* ®700603** Cefpodoxime 23-28 mm / 9-16 mm Ceftazidime 25-32 mm / 10-18 mm Aztreonam 28-36 mm / 9-17 mm Cefotaxime 29-35 mm / 17-25 mm Ceftriaxone 29-35 mm / 16-24 mm	When performing the ESBL confirmatory tests, *K. pneumoniae* ATCC® 700603 and E. *coli* ATCC® 25922 should be tested routinely (e.g. weekly or daily). ***E.coli* ATCC® 25922**: < 2-mm increase in zone diameter for antimicrobial agent tested alone versus its zone when test in combination with clavulanic acid. ***K. pneumoniae* ATCC® 700603:** ≥5-mm increase in ceftazidime-clavulanic acid zone diameter vs ceftazidime alone; 3-mm increase in cefotaxime -clavulanic acid zone diameter vs cefotaxime alone

Note: If ceftazidime-clavulanate (30 µg/10 µg) disks are prepared in house, then use dry disks immediately after preparation. Discard if not used and do not store disks.

Table 8.1b: Screening and confirmatory tests for ESBLs in *Klebsiella pneumoniae, K. oxytoca*, and *Escherichia coli* (MICs)

Method	**Initial screen test**	**Phenotypic confirmatory test**
Medium	**Cation-adjusted Mueller-Hinton broth (CAMHB)**	**CAMHB**
Antimicrobials	For *K. pneumoniae, K. oxytoca, E. coli:* Cefpodoxime 4 µg/mL or Ceftazidime 1 µg/mL or Aztreonam 1 µg/mL or Cefotaxime 1 µg/mL or Ceftriaxone 1 µg/mL (The use of more than one antimicrobial agent for screening improves the sensitivity of detection).	Ceftazidime 0.25–128µg /mL Ceftazidime-clavulanic acid 0.25/4–128/4 µg/mL And Cefotaxime 0.25–64 µg/mL Cefotaxime-clavulanic acid 0.25/4–64/4 µg/mL (Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid).
Inoculum Incubation length and conditions	Standard broth dilution recommendations as described above	Standard broth dilution recommendations as described above
Results	Growth at or above the concentrations listed may indicate ESBL production (i.e., for *E. coli, K. pneumoniae, K. oxytoca* : MIC ≥8 µg/mL for cefpodoxime or MIC ≥ 2 µg/mL for ceftazidime, aztreonam, cefotaxime, or ceftriaxone).	A ≥ 3 twofold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanate vs the MIC of the agent when tested alone = ESBL (e.g., ceftazidime MIC = 8 µg/mL; ceftazidime-clavulanate MIC = 1µg/mL).
QC recommendation	When testing ESBL-screening antimicrobial agents, *K. pneumoniae* ATCC® 700603 should be used for quality assessment (e.g. training, competency, or test evaluation). Either strain, *K. pneumoniae* ATCC® 700603 or *E. coli* ATCC® 25922 should be used for routine QC (e.g. weekly or daily). ***E. coli* ATCC® 25922**: No growth ***K. pneumoniae* ATCC® 700603**:Growth Cefpodoxime ≥ 8 µg/mL Ceftazidime ≥ 2 µg/mL Aztreonam ≥ 2 µg/mL Cefotaxime ≥ 2 µg/mL Ceftriaxone ≥ 2 µg/mL	When performing the ESBL confirmatory tests, *K. pneumoniae* ATCC® 700603 and *E. coli* ATCC® 25922 should be tested routinely (e.g. weekly or daily). ***E.coli* ATCC® 25922**: < 3 twofold concentrate -ion decrease in MIC for antimicrobial agent tested in combination with clavulanate vs the MIC of the agent when tested alone. ***K. pneumoniae* ATCC® 700603:** ≥ 3 twofold concentration decrease in an MIC for an antimicrobial agent tested in combination with clavulanate vs the MIC of the agent when tested alone.

8.1.1 ESBL confirmatory tests[20]

Phenotypic confirmatory testing depends on demonstrating a synergy between clavulanic acid and an indicator cephalosporin. These tests distinguish AmpC β-lactamases (not inhibited by β-lactamase inhibitors) from ESBLs. Numerous variations of confirmatory testing have been described but few are convenient for routine use: combined disk method, double disk (DD) approximation test, MIC methods, and E-test ESBL strips.

Note: The CLSI recommends the combined disk method and the MIC method for ESBL confirmation and the same should be followed.

8.1.1.1 Combined disk test[20]

The combined disk method depends on comparing the inhibition zones around disks containing an indicator cephalosporin with and without clavulanic acid. As per CLSI guidelines, 10 µg of clavulanic acid should be added to each of a cefotaxime (30 µg) and a ceftazidime (30 µg) disk. If ESBL is produced, the zone diameters given by the disks with clavulanate should be >5 mm larger than disk tested alone (without inhibitor). A variation of the combination disk method is based on comparing the zone given by cefpodoxime (10 µg) and cefpodoxime/clavulanate (10 mg + 1 µg). ESBL production should be inferred if the zones given by the disks with clavulanate are >5 mm larger than those without the inhibitor.

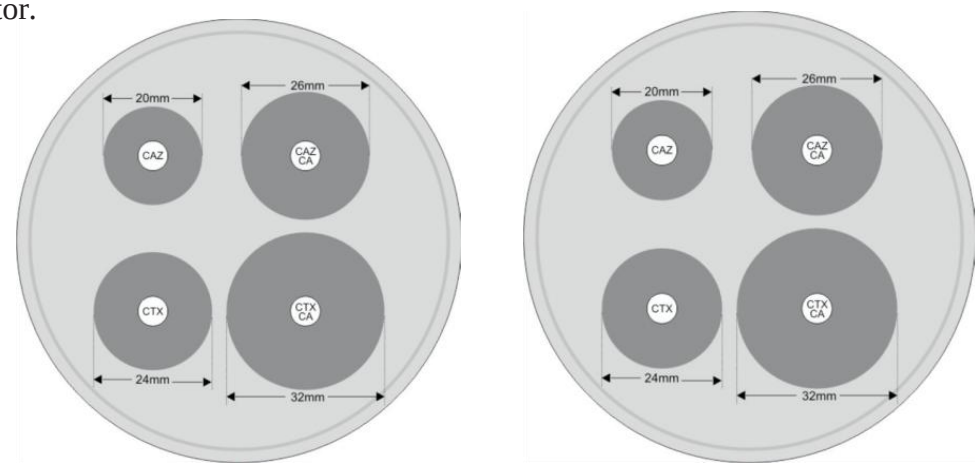

Fig 8.1: Combined disk test

8.1.1.2 Double disk approximation test (DD)

Inoculate the plate as described for a standard disk diffusion test. Place disks containing aztreonam and expanded-spectrum cephalosporins at 30 mm (center to center) from an amoxicillin-clavulanate or clavulanic acid (10 mg) disk. Incubate overnight at 35 °C. Infer the production of an ESBL by test organism as positive if characteristic distortions/expansions of the inhibition zones are present towards the clavulanate disk which indicates the clavulanate potentiation of the activity of test drug. Repeat negative double disk tests with a disk spacing of 20 mm (center to center)[21].

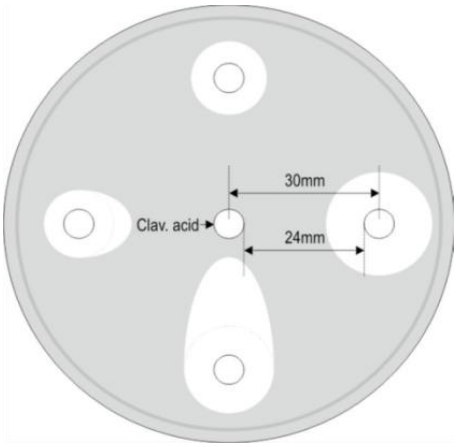

Fig 8.2: Double disk approximation test

8.2 Detection of AmpC-β-lactamases

8.2.1 AmpC disk test[22]

The test is based on the use of tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment. Prepare AmpC disks (filter paper disks containing tris-EDTA) in-house by applying 20 µl of a 1:1 mixture of normal saline and 100X tris-EDTA (1.0 M tris-HCl, 0.1 M EDTA, pH 8.0, filter sterilized) to sterile filter paper disks. Allow the disks to dry and store at 2-8 °C. Inoculate surface of a MHA plate with a lawn of *E. coli* ATCC® 25922 according to standard disk diffusion method. Immediately prior to use, rehydrate AmpC disks with 20 µl of distilled water, and apply several colonies of each test organism to the disk. Place a 30 µg cefoxitin disk on the inoculated surface of the MHA. Place the inoculated AmpC disk face in contact with the agar surface almost touching the antibiotic disk. Invert the plate and incubate overnight at 35 °C in ambient air. Examine plates for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicative of no significant inactivation of cefoxitin (negative result).

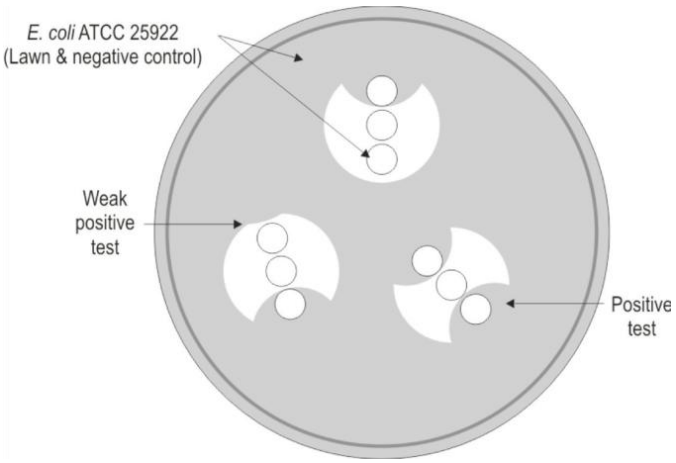

Figure 8.3: AmpC disk test

8.3 Detection of Metallo-β-lactamases (MBL)[20]

8.3.1 EDTA-disk synergy (EDS) test

Dilute the overnight Luria Bertani broth culture of the test isolate to a turbidity of 0.5 McFarland standard and spread on the surface of a MHA plate. Place a disk containing 10 µg of imipenem (IPM) on the surface, and a second disk containing 10 µl of 0.5 M EDTA 15 mm (edge-to-edge) from the first disk. Incubate overnight at 37 °C and interpret the presence of an expanded growth inhibition zone between two disks as positive for synergy.

8.3.2 Extended EDS (eEDS) test

The sensitivity of the EDS can be increased by certain modifications. EDS that includes both the following modifications named as the extended EDS (eEDS) include the following:

(i) The incorporation of meropenem (MEM) and ceftazidime (CAZ) to the original assay,
(ii) Parallel test employing a disk with a lower EDTA concentration (10 µl of 0.1 M EDTA) placed 10 mm, edge to edge, from the other disk.

The use of various β-lactam and EDTA concentrations increases the EDS sensitivity.

(a) The use of only IPM in the EDS may fail to detect MBL producers, clear synergistic zones can be observed upon incorporation of MEM and CAZ to the assay.
(b) The use of 10 µl of 0.5 M EDTA may result in undesirable large growth inhibition zones in some cases making MBL detection difficult.
(c) The problem can be avoided by reducing the EDTA amount i.e. use of 10 µl of 0.1 M EDTA result in clear synergy zones and revealing MBL production.

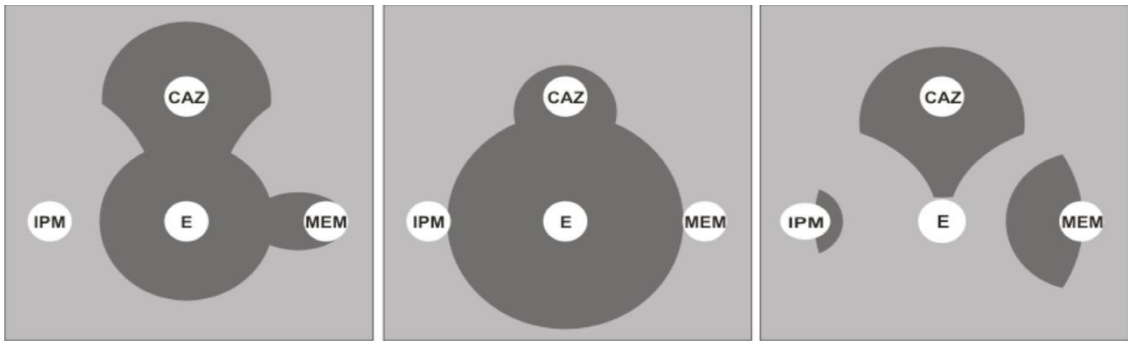

Figure 8.4: EDTA-disk synergy test

8.3.3 Double-disk synergy test (DDST) using mercaptopropionic acid (MPA)

Inoculate 0.5 McFarland bacterial suspension on a MHA plate. Place filter disks containing 5 µl of the chosen mercaptopropionic acid (MPA) inhibitor solution on the MHA plate. Align the IPM disks around the MPA inhibitor disk already placed on the MHA plate. The distance between the inhibitor and substrates should be 2.0 cm (from center to center). The

appearance of either an enhanced or a phantom zone between the antimicrobial agents and the inhibitor disk should be considered as a positive result and indicative of MBL production.

8.3.4 Combined disk method (CD)

Add 0.5 M EDTA solution (pH 8.0) to 10 µg IPM disk to obtain an EDTA content of 750 µg per disk. Immediately dry the disk in an incubator and store at 4 °C. Adjust inoculum of the test strain to McFarland 0.5 standard, and inoculate to MHA plate. Place a 10 µg IPM disk, an IPM plus 750 µg EDTA disk and an EDTA disk (750 µg) as a control on MHA plate. After overnight incubation, the established zone diameter difference of ≥7 mm between IPM disk and IPM plus EDTA should be interpreted as EDTA synergy positive.

8.4 Carbapenemase detection

Initially, screen the isolates by disk diffusion test using IPM (10 µg) and meropenem (10 µg). Further test the isolates resistant to either of the two with a zone of inhibition of ≤ 19 mm or MIC of 2–4 µg/ml using phenotypic tests namely modified carbapenem inactivation method (mCIM) with or without eCIM, CarbaNP test, and/or a molecular assay.

8.4.1 Carba NP Test

Prepare solution 'A' of 0.5% w/v phenol red (0.5 g of phenol red in 100 ml double distilled autoclaved water, adjust the pH to 7.8 by adding 0.1N NaOH) and 0.1 mM $ZnSO_4$ (180 µl of 10 mM $ZnSO_4$). Store the solution at 4-8 °C. Prepare solution 'B' by adding 6 mg of IPM to 1 ml of solution A. It is advisable to weigh out at least 10 mg of powder. Store the solution at 4-8 °C for upto 3 days. For one test 100 µl of each solution is required and accordingly the volume of solutions may be scaled up depending on the number of tests to be performed. Label two 1.5 ml centrifuge tubes as A and B. Take. Add 100 µl of 20 mM Tris-HCl lysis buffer (pH 7.4) in each of two tubes. Emulsify a 1 µl loopful of bacterial culture from overnight blood agar plate of the test isolate(s) and suspend the bacterial isolate(s) in each tube, and vortex for 1 minute. Add 100 µl of solution A to tube A and 100 µl of solution B to tube B. Incubate the tubes at 35 °C for upto 2 hours and observe for color change. Interpret test as CarbaNP negative if both the tubes remain red or red-orange after 2 hours. Interpret test as CarbaNP positive if tube A remains red or red-orange, while tube B turns light orange, yellow or dark yellow. Interpret test as invalid if both the tubes turn yellow. A positive result implies the tested isolate as a carbapenemase producer and vice versa.

Note: With every panel of test isolates, *K. pneumoniae* ATCC® BAA-1705 and *K. pneumoniae* ATCC® BAA-1706 should be used as positive and negative controls respectively.

8.4.2 Modified carbapenem inactivation method (mCIM)

Emulsify 1 µl of test organism with a sterile inoculating loop and inoculate into the 2 ml of tryptic soy broth (TSB) in a tube. Mix by vortexing for 10-15 seconds. Add a 10-µg meropenem (MEM) disk aseptically into the bacterial suspension. Incubate for 4 hours (±15 minutes) at 35 °C (± 2 °C) in ambient air. Just prior to completion of the 4 hours, prepare a suspension (0.5 McFarland standard) of the mCIM indicator organism i.e. *E. coli* ATCC® 25922 (carbapenem-susceptible strain) using the colony suspension method. Inoculate the surface of MHA with indicator organism suspension plate following the procedure for standard disk diffusion test. Remove the MEM disk from the TSB bacterial suspension using a 10 µl inoculating loop dragging the loop along the edge of the tube to remove excess liquid. Place the disk onto the inoculated MHA plate and incubate the plates for 18-24 hours at 35 °C (±2 °C) in ambient air.

mCIM zone diameter of 6-15 mm should be interpreted as positive result, while 16-18 mm as an indeterminate result (requiring further testing to establish the presence or absence of carbapenemase production), and ≥19 mm as a negative result. Positive result with either of the two tests gives indication of carbapenemase production. The strain *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-2146 is used as the positive control for serine carbapenemase and metallo-β-lactamase producers. In addition, *K. pneumoniae* ATCC BAA-1706 is used as the negative control.

8.5 Special AST of *S. aureus* and CoNS

8.5.1 Detection of inducible clindamycin resistance

Macrolide-resistant strains of *S. aureus, S. lugdunensis* and CoNS spp. may express constitutive or inducible resistance to clindamycin or resistance only to macrolides. A disk diffusion test or a single well test using broth microdilution can be used for detecting inducible clindamycin resistance.

8.5.1.1 Disk diffusion test (D-zone test)

A standard blood agar plate may be used for the inoculum purity check. Place the clindamycin disk (2 µg) at a distance of 15-26 mm away from the edge of an erythromycin (15µg) disk on MHA plate. Incubate the plate at 35 ^{o}C for 18 hours. Check for a D-shaped blunting in the clindamycin inhibition zone adjacent to the erythromycin disk or hazy growth within the zone of inhibition around clindamycin which indicates inducible clindamycin resistance. If no D-shaped blunting zone is observed, then it should be reported as susceptible or intermediate to clindamycin.

8.5.1.2 Broth microdilution test

The broth microdilution test applies only to isolates that are erythromycin resistant (MICs

≥ 8 µg/ml) and clindamycin susceptible or intermediate (MICs ≤ 2 µg/ml). Cation adjusted MHB (CAMHB) media should be used for standard broth microdilution procedure. Place a combination of erythromycin (4 µg/ml) and clindamycin (0.5 µg/ml) together in a single well. Incubate at 35 ^{o}C for 18-24 hours. Following incubation, growth in the well indicates the presence of inducible clindamycin resistance. Organisms that grow in the microdilution well or that show D-zone should be reported as clindamycin resistant.

8.5.1.3 QC recommendation

S. aureus ATCC® BAA-977 should demonstrate (positive) inducible clindamycin resistance whereas, *S. aureus* ATCC® BAA-976 should not demonstrate (negative) inducible clindamycin resistance. *S. aureus* ATCC® 25923 should be used for routine QC of clindamycin and erythromycin disks, whereas *S. aureus* ATCC® BAA-976 or *S. aureus* ATCC® 29213 (no growth) should be used for routine QC of broth microdilution.

8.6 Detection of MRSA

The confirmed *S. aureus* and CoNS isolates should be checked for phenotypic and genotypic confirmation for detection of methicillin resistance. Except for active cephalosporins like cefatroline, MRSA usually show resistance to all β-lactam agents namely cephalosporins and carbapenem. MRSA detection is based on the phenotypic resistance to cefoxitin, oxacillin and latex agglutination tests for detection of penicillin-binding protein (PBP2a). Cefoxitin is used as surrogate for the detection of MRSA for oxacillin or methicillin resistance. Both cefoxitin disk diffusion (DD) test and MIC to cefoxitin (broth microdilution) can be used to detect oxacillin resistance among *S. aureus*. Cefoxitin disk diffusion test is preferred for most of the CoNS, however for *S. pseudintermedius* and *S. schleiferi*, disk diffusion test should be performed using oxacillin disk (1 µg). To avoid any discrepancy, the use of cefoxitin (and oxacillin wherever applicable) disk diffusion and oxacillin salt-agar screening-plate for detection of MRSA should be preferred.

Table 8.2: Methods for detecting oxacillin resistance in staphylococci

	Oxacillin MIC	**Oxacillin DD**	**Cefoxitin MIC**	**Cefoxitin DD**	**Direct Methods (for *mecA* or PBP2a)**
S. aureus	Yes	No	Yes	Yes	Yes
S. lugdunensis	Yes	No	Yes	Yes	Yes
S. pseudintermedius *S. schleiferi*	Yes	Yes	No	No	Yes
CoNS (except *S. lugdunensis*)	Yes	No	No	Yes	Yes

Note: NaCl (2% w/v; 0.34 mol/l) supplementation required for both agar and broth dilution testing of oxacillin.

8.6.1 Disk diffusion test (DD)

Follow standard disk diffusion testing conditions and incubate at 35 °C for 18 hours for *S. aureus* and 24 hours for CoNS.

Cefoxitin (30 µg): Test for *S. aureus, S. lugdunensis* and CoNS other than *S. pseudintermedius* and *S. schleiferi*. For *S. aureus* and *S. lugdunensis,* cefoxitin zone of ≤21 mm should be reported as oxacillin resistant and a zone of ≥22 mm should be reported as oxacillin susceptible. For CoNS, disk diffusion zones of ≤24 mm should be reported as oxacillin resistant, whereas zones of ≥25 mm should be reported as oxacillin susceptible.

Oxacillin (1 µg): For *S. pseudintermedius* and *S. schleiferi,* disk diffusion zones of ≤17mm and ≥18 mm should be reported as oxacillin resistant and oxacillin susceptible respectively. Besides, following MIC may also be considered for the determination of *mecA* mediated methicillin resistance *S. aureus* and CoNS.

Table 8.3: MIC range for methicillin resistance *S. aureus* and CoNS

Antimicrobial agent (MIC dilution range)	Organism	MIC (µg/ml)	
		Sensitive	Resistant
Cefoxitin (0.016-256 µg/ml)	*S. aureus* *S. lugdunensis*	≤4	≥8
Oxacillin (0.016-256 µg/ml)	*S. pseudintermedius*	≤0.25	≥0.5

8.6.2 Oxacillin Agar Screen

Oxacillin salt-agar screening-plate procedure should be used for the detection of MRSA only. Inoculate direct colony suspension (0.5 McFarland standard) of *S. aureus* onto MHA supplemented with NaCl (4% w/v; 0.68 mol/L) and 6 µg oxacillin/ml. Incubate the plate at 35 °C for 24 hours and examine carefully for small colonies (> 1 colony) or a light film of growth, indicating oxacillin resistance.

If any discrepancy is observed between the results of cefoxitin disk diffusion and oxacillin salt screen agar in detection of MRSA, latex agglutination test for PBP2a or *mec*A PCR may be performed for confirmation, if facilities are available.

S. aureus ATCC® 43300 (resistant) should be used for QC of disk diffusion test, MIC testing and oxacillin salt agar. *S. aureus* ATCC® 25923 may be used for routine QC of (negative) oxacillin salt agar.

8.6.3 Vancomycin Agar Screen

Used for the detection of *S. aureus* with resistance or reduced susceptibility to vancomycin. This method is not recommended for CoNS. For the isolates which grow on vancomycin agar, determine vancomycin MIC using a validated MIC method (E-strip or broth micro dilution). *E. faecalis* ATCC® 29212 should be used for routine QC (susceptible) of MIC method.

Table 8.4: Screening tests for oxacillin resistance and reduced susceptibility to vancomycin in *S. aureus*

Screen test	Oxacillin resistance	Reduced susceptibility to Vancomycin
Medium	MHA with NaCl (4% w/v; 0.68 mol/l)	Brain Heart Infusion (BHI) agar
Antimicrobial concentration	6 µg/ml oxacillin	6 µg/ml vancomycin
Inoculum	Direct colony suspension to obtain 0.5 McFarland turbidity. Dip 1 µl loop in the suspension and spot an area 10-15 mm in diameter. Alternatively, dip a swab in the suspension and express excess liquid, spot a similar area or streak an entire quadrant.	Direct colony suspension to obtain 0.5 McFarland turbidity. Preferably, using a micropipette, spot a 10-µl drop onto agar surface. Alternatively, dip a swab in the suspension and express excess liquid, spot an area 10-15 mm in diameter or streak a portion of the plate.
Incubation conditions and incubation period	35 °C; ambient air 24 hours	35 °C; ambient air 24 hours
Results	> 1 colony = resistant Examine carefully with transmitted light for > 1 colony or light film of growth.	> 1 colony = presumptive reduced susceptibility Examine carefully with transmitted light for > 1 colony or light film of growth. Perform vancomycin MIC using a validated MIC method to confirm the reduced susceptibility.
QC Recommendations	*S. aureus* ATCC® 29213 – Susceptible *S. aureus* ATCC® 43300 – Resistant	*E. faecalis* ATCC® 29212 – Susceptible *E. faecalis* ATCC® 51299 – Resistant

8.7 Molecular typing for methicillin resistant *S. aureus*

The phenotypically identified *S. aureus* and CoNS isolates (or in any case of discrepancy in results) should be checked for the presence of methicillin resistance gene(s), i.e. *mec*A, *mec*B and *mec*C by PCR.

Note: It is recommended to screen all the confirmed *S. aureus* and CoNS isolates for *mec*A and *mec*C gene at least for the confirmation of methicillin resistance.

Table 8.5: Primers and PCR cycle for the detection of *mecA, mecB* and *mecC* genes

Gene	Primers (5'-3')	Amplicon (bp)	Programme	Reference
mecA	F: TCCAGATTACAACTTCACCAGG	162	94 ºC for 5 minutes; 30 Cycles of 94 ºC for 30 seconds, 59 ºC for 1 minutes, 72 ºC for 1 minutes; final extension 72 ºC for 10 minutes	23
	R: CCACTTCATATCTTGTAACG			
mecC	F: GAAAAAAAGGCTTAGAACGCCTC	138		
	R: GAAGATCTTTTCCGTTTTCAGC			
mecB	F: TTAACATATACACCCGCTTG		95 °C for 5 minutes; 35 cycles of 95 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 2.5 minutes; final extension: 72 ºC for 7 minutes	24
	R: TAAAGTTCATTAGGCACCTCC			

8.8 Molecular detection of vancomycin resistant *S. aureus*

The isolates that do not show susceptibility to vancomycin in the phenotypic screening tests may further be tested for the presence of vancomycin resistant gene(s) like *vanA* and *vanB*.

Table 8.6: Primers for detection of vancomycin resistant gene(s)

Gene	Primers	Amplicon (bp)	Reference
vanA	F: GGG AAA ACG ACA ATT GC	723	25
	R: GTA CAA TGC GGC CGT TA		
vanB	F: ACGGAATGGGAAGCCGA	647	
	R: TGCACCCGATTTCGTTC		

8.9 Special AMST for *Enterococcus*

8.9.1 Identification of vancomycin resistant *Enterococcus* (VRE)

8.9.1.1 Vancomycin Screening Agar

BHI agar supplemented with 6 µg/ml of vancomycin should be used for the identification of VRE isolates as per following method.

i. Dissolve 9.3 g dehydrated BHI media in 180 ml of double distilled water.
ii. Prepare stock solution 1 (S1) of 60 mg/10 ml (6 µg/ml) of vancomycin.
iii. Add 1 ml of S1 to 9 ml of sterile distilled water to make stock solution 2 (S2).

iv. Add 18 ml of sterile distilled water to 2 ml of S2 to make the working solution.
v. Add 20 ml of the working solution to 180 ml of the molten BHI agar and pour 20 ml agar into petri dishes.
vi. Suspend the test strain of *Enterococcus* in nutrient broth with a turbidity corresponding to 0.5 MacFarland standard. Spot inoculate on to the agar using a 10 µl loop. Alternatively, dip a swab in the suspension and express excess liquid, spot an area 10-15 mm in diameter or streak an entire quadrant.
vii. Include positive control (*E. faecalis* ATCC® 51299; resistant) and negative control (*E. faecalis* ATCC® 29212; susceptible) on each plate.
viii. Incubate for 24 hours at 37 °C.
ix. Appearance of even a single colony or film of growth indicates vancomycin resistance.

8.9.1.2 MIC for vancomycin by agar dilution method

Perform vancomycin MIC on *Enterococcus* spp. (such as *E. gallinarum, E. casseliflavus,* or *E. flavescens*) that grows on vancomycin screening agar. Perform MIC using BHI agar incorporated with vancomycin in concentrations ranging from 1 µg/ml to 32 µg/ml. Prepare the vancomycin stock solutions at ten times the concentrations to be tested and make twofold dilutions of these stock solutions as per CLSI guidelines.

i. Add 2 ml of the vancomycin solution to 18 ml of molten BHI agar at 50 ºC and allow the agar to solidify.
ii. Inoculate the strains to be tested, in sterile normal saline to make a suspension with turbidity equivalent to 0.5 McFarland standard.
iii. Using 10 µl loop, inoculate a spot of the suspension on plates of different concentrations of vancomycin.
iv. Incubate the plates at 35 ºC for 24 hours. Always include an antibiotic free control medium to check the viability of the strains. Use *E. faecalis* ATCC® 29212 as QC for determining MIC values.
v. Examine the plates both in reflected and transmitted light for determining MIC (lowest concentration that shows no growth) of vancomycin.

8.10 Detection of High-Level Aminoglycoside Resistance (HLAR) in *Enterococcus* spp.

Standard disk diffusion (DD) or broth or agar dilution (MIC) procedures using BHI should be used for detection of gentamicin and streptomycin HLAR in *Enterococcus* spp. Perform an agar dilution or broth dilution MIC test to confirm, if disk diffusion test result (7-9 mm) is inconclusive. Incubate at 35 ºC for 16-18 hours for DD and 24-48 hours for MIC testing. Isolate should be interpreted as resistant if any growth in broth or single colony on agar is observed in MIC testing.

Table 8.7: Interpretation of screening test for HLAR in *Enterococcus* spp

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC breakpoints (µg/ml)	
		S	I	R	S	R
Gentamicin HLAR	120	≥ 10	7-9	6	≤ 500	> 500
Streptomycin HLAR	300	≥ 10	7-9	6	≤ 1000 (broth) ≤ 2000 (agar)	> 1000 (broth) > 2000 (agar)

Note:

(i) For QC of HLAR DD screen tests, use *E. faecalis* ATCC® 29212 (gentamicin: 16-23 mm; streptomycin: 14-20 mm).

(ii) For MIC, use *E. faecalis* ATCC® 29212 (susceptible) and *E. faecalis* ATCC® 51299 (resistant) as controls.

Also, test *Enterococcus* isolates that grows on vancomycin screening agar for motility and pigment production to distinguish species with acquired resistance (e.g. *vanA* and *vanB*) from those with intrinsic, intermediate-level resistance to vancomycin (e.g. *vanC*), such as *E. gallinarum*, *E. casseliflavus*, or *E. flavescens.*

Molecular Detection of Antimicrobial Resistance

CHAPTER 9
MOLECULAR DETECTION OF ANTIMICROBIAL RESISTANCE IN ISOLATES

9.1 Molecular detection of β-lactamase producers

The following primer sequences should be used for the PCR based screening to detect ESBL, MBL or *Amp*C producers[26].

Table 9.1: Target genes with primers to detect ESBL, MBL or *Amp*C producers

Details	Target genes	Sequence (5'-3')	Annealing temp. (°C)	Amplicon size (bp)	ATCC/ NCTC strains
Multiplex I TEM, SHV and OXA-1-like	TEM variants including TEM-1 and TEM-2	CATTTCCGTGTCGCCCTTATTC	60	800	*K.pneumonae* BAA2783 (TEM-1)
		CGTTCATCCATAGTTGCCTGAC			*Proteus mirabilis* BAA2792 (TEM-2)
	SHV variants including SHV-1	AGCCGCTTGAGCAAATTAAAC		713	*K.pneumoniae* BAA2783
		ATCCCGCAGATAAATCACCAC			
	OXA-1, OXA-4 and OXA-30	GGCACCAGATTCAACTTTCAAG		564	*E.coli* BAA2779 (OXA-1)
		GACCCCAAGTTTCCTGTAAGTG			
Multiplex II CTX-M group 1, group 2 and group 9	Variants of CTX-M group 1 including CTX-M-1, CTX-M-3 and CTX-M-15	TTAGGAARTGTGCCGCTGYA[a]		688	*E.coli* NCTC13461 (common for group 1)
		CGATATCGTTGGTGGTRCCAT[a]			
	Variants of CTX-M group 2 including CTX-M-2	CGTTAACGGCACGATGAC		404	*E.coli* NCTC13462 (common for group 2)
		CGATATCGTTGGTGGTRCCAT[a]			
	Variants of CTX-M group 9 including CTX-M-9 and CTX-M-14	TCAAGCCTGCCGATCTGGT		561	*E.cloacae* NCTC13464 (common for group 9)
		TGATTCTCGCCGCTGAAG			
CTX-M group 8/25	CTX-M-8, CTX-M-25, CTX-M-26 and CTX-M-39 to CTX-M-41	AACRCRCAGACGCTCTAC[a]		326	*E.coli* NCTC 13463 (CTXM-8)
		TCGAGCCGGAASGTGTYAT[a]			*K.pneumoniae* NCTC13465 (CTX-

					M 25)
Multiplex III ACC, FOX, MOX, DHA, CIT and EBC	ACC-1 and ACC-2	CACCTCCAGCGACTTGTTAC	60	346	To be confirmed by sequence analysis of the amplicon
		GTTAGCCAGCATCACGATCC			
	FOX-1 to FOX-5	CTACAGTGCGGGTGGTTT		162	
		CTATTTGCGGCCAGGTGA			
	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11, CMY-19	GCAACAACGACAATCCATCCT		895	
		GGGATAGGCGTAACTCTCCCAA			
	DHA-1 and DHA-2	TGATGGCACAGCAGGATATTC		997	
		GCTTTGACTCTTTCGGTATTCG			
	LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18, CMY-21 to CMY-23	CGAAGAGGCAATGACCAGAC		538	*E.coli* BAA 2779 (CMY-2) *Proteus mirabilis* BAA 2791 (CMY-4) *Proteus mirabilis* BAA 2792 (CMY-16)
		ACGGACAGGGTTAGGATAGY[a]			
	ACT-1 and MIR-1	CGGTAAAGCCGATGTTGCG		683	To be confirmed by sequence analysis of the amplicon
		AGCCTAACCCCTGATACA			
Multiplex IV GES, PER, and VEB	GES-1 to GES-9, and GES-11	AGTCGGCTAGACCGGAAAG		399	*P. aeruginosa* BAA 2794 (GES-5)
		TTTGTCCGTGCTCAGGAT			
	PER-1 and PER-3	GCTCCGATAATGAAAGCGT		520	*A. baumannii* 2801 (PER1)
		TTCGGCTTGACTCGGCTGA			
	VEB-1 to EB-6	CATTTCCCGATGCAAAGCGT		648	To be confirmed by sequence analysis of the amplicon
		CGAAGTTTCTTTGGACTCTG			

[a:] Y=T or C; R=A or G; S=G or C

***** For all wherever the specific positive control strains are not available the sequence analysis of the amplicons needs to be used. Based on sequence identification internal control strains may be generated for future use.

9.2 Molecular detection of methicillin resistance

The uniplex PCR based molecular screening should be performed for methicillin resistant genes *mec*A, *mec*B and *mec*C using following primer sequences.

Table 9.2: Primer sequences to detect methicillin resistant genes

Gene	Sequence(5'-3')	Annealing temp. (°C)	Amplicon size (bp)	ATCC/ NCTC strains	References
*mec*A	'F' ACGAGTAGATGCTCAATATAA	56	293	*S. aureus* BAA1683 *S. aureus* BAA2094 *S. aureus* BAA 42	27
	'R' CTTAGTTCTTTAGCGATTGC				
*mec*B	'F' TTAACATATACACCCGCTTG	57	2264	*Macrococcus caseolyticus 13548*	24
	'R' TAAAGTTCATTAGGCACCTCC				
*mec*C	'F' GCTCCTAATGCTAATGCA	50	304	*S. aureus* BAA2313	28
	'R' TAAGCAATAATGACTACC				

9.3 Molecular detection of vancomycin resistance

The presence of vancomycin resistant gene(s) like *van*A and *van*B should be detected by multiplex PCR using following oligonucleotides as described.

Table 9.3: Primer sequences to detect vancomycin resistant genes

Gene	Sequence(5'-3')	Annealing temp. (°C)	Amplicon size (bp)	ATCC/ NCTC strains	References
*van*A	'F' GGGAAAACGACAATTGC	45	732	*E. faecium* BAA 2320	25
	'R' GTACAATGCGGCCGTTA				
*van*B	'F' ACGGAATGGGAAGCCGA	45	647	*E. faecalis* BAA 2365	25
	'R' TGCACCCGATTTCGTTC				

9.4 Molecular detection of carbapenamase producers

Carbapenem resistant Enterobacteriaceae (CRE) includes the *Klebsiella pneumoniae* carbapenemase (KPC) producers and New Delhi Metallo-β-lactamases (NDM-1, NDM-2) producers. Apart from Enterobacteriaceae, KPC and NDM enzymes are also detected in *Pseudomonas*. These enzymes break down the carbapenem class of drugs. Other enzymes include Verona Integron-mediated Metallo-β-lactamase (VIM), Guiana extended spectrum β-lactamases (GES), Oxacillinase-type carbapenemases (OXA-48 and its variants) and imipenemases (IPM). Various carbapenemase producing genes *viz*., *bla*NDM, *bla*KPC, *bla*OXA-48 and its variants, *bla*GES, *bla*IMP and *bla*VIM should be screened for the detection of carbapenem resistance[29]. Reported reference strains[29,30]

should be used as positive control *viz*., *K. pneumoniae* ATCC® BAA21246 for *bla*NDM, *K. pneumoniae* ATCC® BAA1705, *K. pneumoniae* ATCC® 13883 for *bla*KPC, *K. pneumoniae* NCTC®13442 for *bla*OXA-48 and its variants, and *E.coli* NCTC® 13476 for *bla*IMP etc.

Table 9.4: Primer sequences to detect carbapenem resistant genes

Gene	Sequence(5'-3')	Annealing temp. (°C)	Amplicon size (bp)	ATCC/ NCTC strains	References
*bla*NDM-1	'F' CAATATTATGCACCCGGTCG	52	632	*K. pneumoniae* BAA21246	31
	'R' CCTTGCTGTCCTTGATCAGG				
*bla*KPC-1	'F' CGTTCTTGTCTCTCATGGCC	52	796	*K. pneumoniae* BAA 1705/ ATCC 13883	30
	'R' CCTCGCTGTGCTTGTCATCC				
*bla*OXA-48	'F' TTGGTGGCATCGATTATCGG	57	743	*K. pneumoniae* NCTC 13442	30
	'R' GAGCACTTCTTTTGTGATGGC				
*bla*IMP	'F' GAAGGYGTTTATGTTCATAC[a]	54	587	*E. coli* NCTC 13476	32
	'R' GTAMGTTTCAAGAGTGATGC[a]				
*bla*VIM	'F' GTTTGGTCGCATATCGCAAC	55	382	*Proteus Mirabilis* BAA2792	32
	'R' AATGCGCAGCACCAGGATAG				

[a:] Y=C or T; M=A or C.

Alternatively, following multiplex PCR[26] can be adopted for the detection of carbapenem resistance.

Table 9.5: Alternate primer sequences to detect carbapenem resistant genes

Details	Target genes	Sequence (5'-3')[26]	Annealing temp. (°C)	Amplicon size (bp)	ATCC/ NCTC strains
Multiplex-I GES and OXA-48-like	GES-1 to GES-9 and GES-11	AGTCGGCTAGACCGGAAAG	57	399	*P. aeruginosa* BAA 2794 (GES-5)
		TTTGTCCGTGCTCAGGAT			
	OXA-48-like	GCTTGATCGCCCTCGATT		281	
		GATTTGCTCCGTGGCCGAAA			
Multiplex II IMP, VIM and KPC	IMP variants except IMP-9, IMP-16, IMP-18, IMP-22 & IMP-25	TTGACACTCCATTTACDG[a]	55	139	*E.coli* NCTC 13476
		GATYGAGAATTAAGCCACYCT[a]			
	VIM variants including VIM-1 and VIM-2	GATGGTGTTTGGTCGCATA		390	*Proteus Mirabilis* BAA2792 (VIM-1)
		CGAATGCGCAGCACCAG			
	KPC-1 to KPC-5	CATTCAAGGGCTTTCTTGCTGC		538	*K. pneumoniae* BAA 1705/ ATCC 13883
		ACGACGGCATAGTCATTTGC			

[a.] Y=T or C; D=A or G or T

9.5 Molecular detection of Quinolone resistance

The plasmid mediated quinolone resistance (PMQR) determinants such as *qnr*A, *qnr*B, *qnr*S, *qep* A and *aac(6')-lb-cr* can be detected for detection of quinolone resistance to class of antibiotics using the primers as follows.

Table 9.6: Primer sequences to detect quinolone resistant genes

Gene	Sequence (5'-3')	Amplicon size (bp)	Annealing temp. (°C)	ATCC/ NCTC strains	References
*qnr*A	'F' ATTTCTCACGCCAGGATTTG	516	56	To be confirmed by sequence analysis of the amplicon	33,34,35
	'R' GATCGGCAAAGGTTAGGTCA				
*qnr*B	'F' GATCGTGAAAGCCAGAAAGG	476	56	*E. cloacae* BAA2806	33,34,35
	'R' ATGAGCAACGATGCCTGGTA				
*qnr*S	'F' GCAAGTTCATTGAACAGGGT	428	57	*E. cloacae* BAA2806	33,34,35
	'R' TCTAAACCGTCGAGTTCGGCG				
qepA	'F' CTGCAGGTACTGCGTCATG	218	60	To be confirmed by sequence analysis of the amplicon	36
	'R' CGTGTTGCTGGAGTTCTTC				
aac(6')-lb-cr	'F' TGCGATGCTCTATGAGTGGCTA	482	57	*E. cloacae* BAA2806	36,37
	'R' CTCGAATGCCTGGCGTGTTT				
*oqx*A	'F' GACAGCGTCGCACAGAATG	339	62	*E. cloacae* BAA2806	36,38
	'R' GGAGACGAGGTTGGTATGGA				
*oqx*B	'F' CGAAGAAAGACCTCCCTACCC	240	62	*E. cloacae* BAA2806	36,38
	'R' CGCCGCCAATGAGATACA				

9.6 Molecular detection of sulphonamide resistance

The sulphonamide resistance (*sul*1) gene can be targeted for detection of resistance to sulphonamide group of drugs using the following primers[39].

Table 9.7: Primer sequences to detect sulphonamide resistant genes

Gene	Sequence(5'-3')	Amplicon size (bp)	Annealing Temp. (°C)	Control strain ATCC	Reference
Sul1	'F' CGGCGTGGGCTACCTGAACG	433	69	*A. baumannii* BAA-29901	39
	'R' GCCGATCGCGTGAAGTTCCG				

9.7 Molecular detection of plasmid-mediated colistin resistance

Molecular detection of plasmid mediated colistin resistance genes namely *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* in Enterobacteriaceae should be carried out using following primer sequences and PCR cycling conditions of initial denaturation at 94 °C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 90 seconds, extension at 72 °C for 60 seconds, and a final extension at 72 °C for 10 minutes.

Table 9.8: Primer sequences to detect plasmid mediated colistin resistance genes

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Annealing Temp. (°C)	Positive control strain	References
mcr-1	'F' AGTCCGTTTGTTCTTGTGGC	320	58	*E.coli* NCTC 13846	40
	'R' AGATCCTTGGTCTCGGCTTG				
mcr-2	'F' CAAGTGTGTTGGTCGCAGTT	715		*Internal controls to be generated*	40
	'R' TCTAGCCCGACAAGCATACC				
mcr-3	'F' AAATAAAAATTGTTCCGCTTATG	929			40
	'R' AATGGAGATCCCCGTTTTT				
mcr-4	'F' TCACTTTCATCACTGCGTTG	1116			40
	'R' TTGGTCCATGACTACCAATG				
mcr-5	'F' ATGCGGTTGTCTGCATTTATC	1644			40
	'R' TCATTGTGGTTGTCCTTTTCTG				

Surveillance Framework

CHAPTER 10

SURVEILLANCE FRAMEWORK

The surveillance framework includes surveillance of antibiotics in food producing animals and food of animal origin. A cluster sampling method should be used for sample collection under surveillance programme.

10.1 Nature and frequency of sampling

a. Surveillance framework includes surveillance of healthy animals [cow, buffalo, pig and poultry (chicken)].
b. Surveillance of diseased animals [cow, buffalo, pig, dog and poultry (chicken)].
c. Single round of surveillance sampling per year in an epidemiological centre/unit.

10.2 Cluster sampling method

a) A cluster sampling method is one of the probability sampling methods which consists of two sampling units. i.e. primary sampling units (PSU) and secondary sampling units (SSU).
b) PSU: Blocks/ Tehsil/ Taluks; PSU are known as clusters.
SSU: Households/individual elements
c) Such sampling strategy is mainly adapted for its feasibility and cost-efficiency, though variance estimation efficiency is compromised.
d) It is one of the most economic forms of sampling, despite the fact that standard errors of the estimates are high as compared to other sampling designs with same sample size.
e) Sampling units consist of a group of population elements rather than just an element in a population. PSU can be selected at random instead of direct random sampling of elements in a population.
f) PSU can be selected by specific element sampling techniques like simple random sampling (SRS), systematic sampling or probability proportional-to-size (PPS) sampling. Similarly, the SSU can be selected using specific element sampling strategy or all SSU can be selected if convenient and feasible.

10.3 Sampling strategy

a. Surveillance sampling can be executed as per 30 cluster sampling model.
b. The 30 clusters can be selected on the basis of PPS sampling strategy.

c. In the following surveillance strategy, sampling should be carried out from 3 blocks/taluks/tehsils (which are known as clusters or PSU) under an epidemiological unit/center/district and each cluster should contain 3-5 numbers of villages.

d. Sampling strategy is depicted (Fig 9.1).

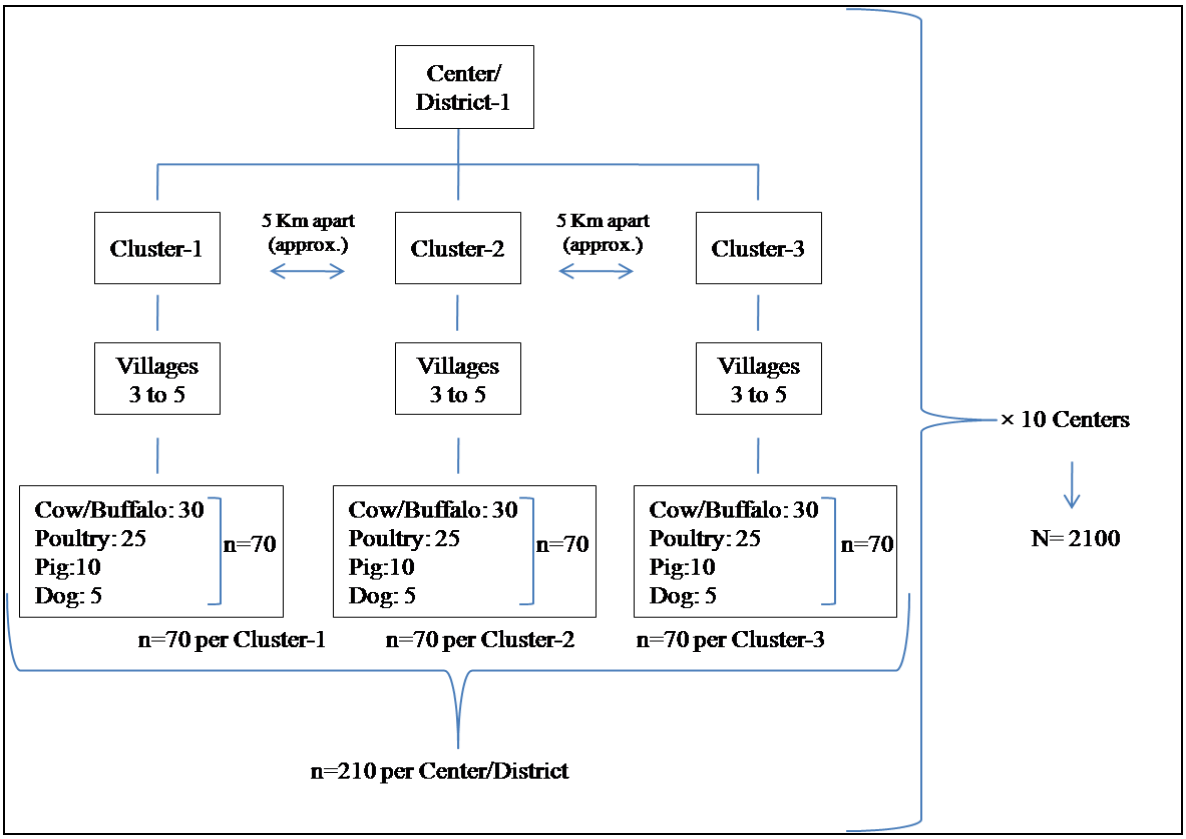

Fig 10.1: Depiction of sampling strategy for surveillance

e. As per sampling strategy depicted above, 10 epidemiological units/centers/districts and 3 clusters are selected under each unit. So, a total 30 clusters are selected for the entire surveillance of AMR.

f. These clusters form the PSUs which includes the blocks/ tehsil/ taluks. Such clusters (n=3) should be selected on the basis of geographical distance (approximately 5 kilometers apart).

g. Within each cluster, three to five villages should be selected.

h. A total 70 samples should be collected from each cluster comprising cow/buffalo (n=30), poultry (n=25), pig (n=10) and dog (n=5). However, sampling plan of these animal species may be relaxed based on the importance of animal domestication in a particular geographical area.

10.4 Additional information (optional): Sampling Model:

	Epidemiological Center/Unit (District)									
					Villages					
					V1	**V2**	**V3**	**V4**	**V5**	
Clusters	**C1**	**Blocks/ Tehsils/ Taluks**	**B1**	**A_1 (N_1)**	a_{11} (n_{11})	a_{12} (n_{12})	a_{13} (n_{13})	a_{14} (n_{14})	a_{15} (n_{15})	**$A_1=a_{11}+a_{12}+a_{13}+a_{14}+a_{15}$ $N_{1=}n_{11}+n_{12}+n_{13}+n_{14}+n_{15}$**
	C2		**B2**	**A_2 (N_2)**	a_{21} (n_{21})	a_{22} (n_{22})	a_{23} (n_{23})	a_{24} (n_{24})	a_{25} (n_{25})	**$A_2=a_{21}+a_{22}+a_{23}+a_{24}+a_{25}$ $N_2=n_{21}+n_{22}+n_{23}+n_{24}+n_{25}$**
	C3		**B3**	**A_3 (N_3)**	a_{31} (n_{31})	a_{32} (n_{32})	a_{33} (n_{33})	a_{34} (n_{34})	a_{35} (n_{35})	**$A_3=a_{31}+a_{32}+a_{33}+a_{34}+a_{35}$ $N_3=n_{31}+n_{32}+n_{33}+n_{34}+n_{35}$**
				$Y=A_1+A_2+A_3$ $X=N_1+N_2+N_3$						

Fig 10.2: Depiction of 30 cluster sampling model

Note:

- C1, C2, C3: Primary Sampling Unit (PSUs)/Clusters
- a11 to a35: No. of SSUs that should be selected from each clusters
- n11 to n35: No. of SSUs in each clusters
- A_1, A_2, A_3 = Total number of households from which samples are collected in a cluster
- $A_1=a_{11}+a_{12}+a_{13}+a_{14}+a_{15}$ =70, where a_{11} to a_{15} are total number of households from which samples are collected in each village under a cluster (C1) or block (B1)
- N_1, N_2, N_3 = Total number of households in a cluster
- $N_{1=}n_{11}+n_{12}+n_{13}+n_{14}+n_{15}$ where n_{11} to n_{15} are total number of households in each village under a cluster (C1) or block (B1)
- $Y=A_1+A_2+A_3$= Total number of households from which samples are collected in an epidemiological unit/center/district
- $X= N_1+N_2+N_3$= Total number of households in an epidemiological unit/center/district

10.4.1 Explanation

- A total of 3 PSUs or Clusters (C: C1 to C3) comprising of blocks (B)/tehsils/taluks i.e. (B1 to B3) respectively are selected under an epidemiological center/unit/district which are approximately 5 km apart comprising of 5 villages (V1 to V5) in each.
- Total SSUs (Households/Farmers) in an epidemiological unit/center/district is 'X' and 'nij (i: 1 to 3 and j: 1 to 5)' are the total SSUs under each PSUs (Clusters or Blocks).
- Suppose, a total of 'Y' SSUs (Households/farmers) need to be selected from total 'X' PSUs, then the weighted average for Cluster (C1) or Block (B1) is calculated as below: **A1=N1(Y/X)**, where N1 is the total SSUs under the Cluster C1 or Block B1
- Similarly, weighted average for a village (V1) under a cluster (C1) is calculated as below: **a11= n11 (A1/N1), where** n11 is the total SSUs in the village V1 under Cluster C1

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ANNEXURES

ANNEXURE I

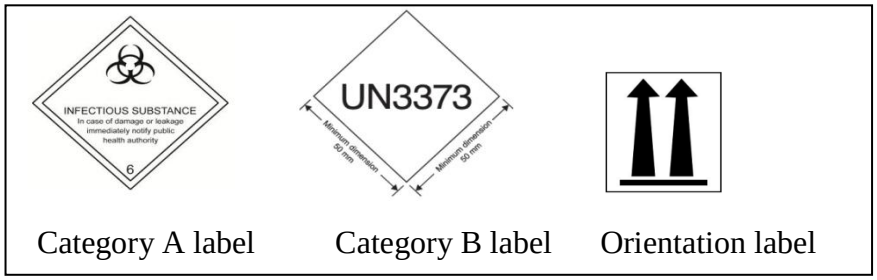
SAMPLE DISPATCH

Introduction

The international regulations for the transport of infectious materials by any mode of transport are based upon the recommendations of the 'United Nations Committee of Experts as per Transport of Dangerous Goods (UNCETDG)'. The recommendations of UN were also followed by the Universal Postal Union (UPU), the International Civil Aviation Organization (ICAO), the International Air Transport Association (IATA) and other alike international transport organizations[41]. Before transporting biological materials, its nature must be determined whether material should be classified as dangerous (hazardous or infectious) goods or not. The infectious substances can be classified as Category A or Category B as per criteria provided in WHO guidance document[41] and assigned UN number accordingly. A category A [UN 2814 (affecting humans) or UN 2900 (affecting animals)] infectious substance is defined as "an infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals". Biological material containing pathogens that do not cause life-threatening disease to humans or animals (do not meet Category A criteria) are assigned to Category B (UN 3373)[41,42].

Packaging, labeling and Dispatch[41,42]

Basic triple packaging system, i.e. a leak-proof primary receptacle, a leak-proof secondary container and an outer box should be used for the shipment of all infectious substances as recommended by UNCETDG. Packages should be marked clearly to provide information about the contents of package, nature of the hazard, UN number and packaging standards applied, and correct address of the consignor and recipient laboratory. Outer packaging box must be labeled appropriately as per approved labels of international standards.



Category A label Category B label Orientation label

Precautions during dispatch[41,42]

- Sample container should be leak-proof, and sealed with adhesive tape, paraffin wax or parafilm. Screw-capped bottles should be used whenever appropriate to avoid the splash during opening of the sample container in laboratory.

- Appropriate refrigerants must be used during transport to stabilize the specimens.
- Certain samples, especially tissue specimens from organs should be transported at cold temperature, whenever applicable, to avoid the event of putrefaction.
- Individual sample container must be packed with proper shock-absorber in an outer container to avoid any spillage or accidental splash during transport.
- Appropriate regulatory issues should be addressed during shipment.
- For overpack dispatch, highest category included in the package should be indicated clearly on the outside of the overpack.
- The documents for shipment should be attached in duplicate inside the plastic envelope, preferably one inside the outer container and another outside to the dispatch parcel.

ANNEXURE II

SAMPLING PROFORMA

(Information to be sent along with sample)

General Information

1. Sample collector's name and affiliation

   (If an investigation team, proceed to Sl. No. 2 of this section)

   _____

   _____

2. Investigation team/Sample collection team and affiliations

   _____

   _____

   _____

3. Date of collection (dd/mm/yyyy) _____ / _____ / _____

   Time _____

4. Place of sample collection *(Indicate the village, block / Tehsil / taluk, district, state etc.)*

   _____

   _____

Epidemiological significance

(Geographical indications)

5. Geographical connotations, if any (Important for occupational/industrial hazards)

   (Indicate below any suggestions for the laboratory personnel's going to process the sample for any epidemiological significance: viz., any hospitals, municipal waste dump yard, animal carcass disposal places, approximation to industrial effluent flows etc.)

- Is there nearby any hospital (human heath/veterinary)? If yes, please indicate below.

_____

- Is there nearby any municipal garbage/waste dump-yard or garbage recycling plant/centre? If yes, please indicate below.

  _____

- Is there any burial yard or animal carcass disposal yard? If yes, please indicate below.

  _____

- Is there any industrial effluent plant nearby to the locality of sample collection? If yes, please indicate below. Is it accessible by any animal or human habitation?

  _____

- Is there any nearby water-body (river/pond/lakes etc) to the locality from where the sample(s) is/are collected? If yes, please indicate below. Is it accessible by any animal or human habitation simultaneously?

  _____

(Climatic conditions)

6. Climatic condition of the place of the sample (if any information available)

- Climatic zone (temperate/tropical) _____
- Latitude and longitude of the area of sampling _____
- Any Geographical Indication System (GIS) _____
- Humidity (%): _____
- Temperature (°C): Min. _____; Max. _____
- Annual Rain fall (range) (mm): _____

(Weather indications)

7. Please indicate the weather prevailing (past/current week) in the locality of the sampling.

- Is there any report of rain-fall recently in the area? If yes, please indicate below.

  _____

- Is there any report of natural calamities (cyclone/floods/heavy blizzard *etc*.) recently in the area? If yes, please indicate below.

  _____

(Earlier disease outbreak history)

8. Is there any disease outbreak/report in the same herd or nearby locality? If yes, please specify.

- Disease outbreak in the same herd (Yes/No) or different herd in same geographical locality _____
- Name of the disease outbreak/reports _____

- Period of the disease outbreak/reports _____
- Morbidity/Mortality _____
- Any treatment regimen (Please specify the antibiotics/antibacterial, if any)
  - Name of the antibiotic/antibacterial _____
  - Doses _____

Sample collection

9. Sample source (veterinary/fisheries/human/environment/food of animal origin)_____ _____

- If sample source is 'veterinary sector', answer the following.

  a. Animal (including birds) species _____
  - If animals, wild/domesticated _____
  - If wild animals, captive/free-ranging _____
  - If birds, wild/domestic _____
  - If wild, captive/migratory birds _____

  b. Age _____

  c. Sex _____

  d. Any previous disease report _____

  e. Sampling technique (invasive/non-invasive)_____ (if sampling from living animal/birds)

  f. If sampling technique is invasive; is the sampling as per the humane procedure (if from living animal/birds) _____

  g. Any Institutional animal ethical committee approval was taken previously for sampling? (Yes/No) _____ (If required)

- If sample source is 'food of animal origin', answer the following.

  a. Food material (raw/processed) _____

  b. Food material (meat/egg) _____

  c. If meat, source of meat (chicken/mutton/chevon/pork/any other, please specify) _____ _____

  d. If fresh water/marine origin food (fish/crab/prawn/shrimps/any other please specify) ____ _____

  e. The place of sampling from food of animal origin is (commercial outlets/road side vendor /butcher shop/slaughter house/any other please specify) _____

10. Nature of the clinical sample (solid, semi-solid or liquid) _____

11. Sample collected in (swab/vial/vacutainer/any other, please specify) _____

12. Type of clinical sample(s) (Please, (√) the appropriate option.)
    - Blood_____; Sputum_____; Milk_____; Pus_____; Saliva_____; Tear_____; Urine_____
    - Faeces_____; Hair_____; Skin tissue materials_____; Fish scales_____; Feathers (in birds)_____; Organ/organelle (tissue)_____
    - Soil_____; Litter material_____; Animal housing dust_____; Water_____; Animal housing sewage/Hospital sewage_____; Air sampling_____
    - Food of animal origin (meat/egg) _____; Food of fresh water/marine origin_____
    - Any other (Please specify)_____
    - If any tissues from organ/organelle, please specify_____

13. Clinical sample is collected from the patient/animals/birds/fish suspected of the pathogen/disease _____ (Please specify the disease/pathogen suspected; if it is a routine sampling, leave it blank.)

Sample Dispatch

14. Date of dispatch (dd/mm/yyyy) _____ / _____ / _____
    Time _____

15. Medium of transport of the sample _____

16. Sample container is labeled as infectious material (both internally in the container as well as externally in the package), (Yes/No) _____

17. Dispatch container/package is marked with 'biohazard sign' (Yes/No) _____
    The sample collection and dispatch performa is duly filled and reviewed before dispatching the container (Yes/No) _____

APPENDICES

APPENDIX I

Bacteriological Media and Reagents Composition

1. Amino acid decarboxylase tests

Use: To determine the ability of bacteria to decarboxylase an amino acid to the corresponding amine with the liberation of carbon dioxide.

Ingredients and preparation

Peptone	5.0 g
Meat extract	5.0 g
Glucose	0.5 g
Pyridoxal	5.0 mg
Bromocresol purple	5.0 ml
Cresol red	2.5 ml
Distilled water	1.0 L

Dissolve the solids in water and adjust the pH to 6.0 before addition of the indicators. This is the basal medium and for testing of decarboxylation reaction, amino acid should be added. Divide the basal medium into four portions and treat as follows:
Add 1% L-lysine hydrochloride.
Add 1% L- ornithine hydrochloride.
Add 1% L- arginine hydrochloride.
No additions (control)
Readjust the pH to 6.0 if necessary with HCL or NaOH. Distribute 1 ml quantities in small tubes containing sterile liquid paraffin to provide a layer about 5 mm thick above the medium.

Procedure

The isolated colony from the test isolate is inoculated in two tubes of each amino acid media, one containing the amino acid and the other to be used as control tube devoid of amino acid. Overlay the tubes with sterile mineral oil to cover about 1cm above the surface and incubate at 35 ºC for 18-24 hours.

Principle and interpretation

This test is based on the ability of some bacteria to decarboxylase an amino acid to the corresponding amine with the liberation of carbon dioxide. The production of these decarboxylases is induced by a low pH and, as a result of their action; the pH rises to neutrality or above. The medium first becomes yellow due to acid production during glucose fermentation; later if decarboxylation occurs, the medium becomes violet. The control should remain yellow.

Result

Amino acid	Positive	Negative
Lysine	*Escherichia coli*	-
	Salmonella	-
Ornithine	*Escherichia coli*	*Klebsiella*
Arginine	*Pseudomonas*	*Escherichia coli*

2. Phenylpyruvic acid medium (PPA)

Use: It is used to determine the ability of an organism to deaminate phenyl alanine to phenyl pyruvic acid enzymatically with resulting acidity.

Ingredients and preparation

Yeast extract	3.0 g
DL-phenylalanine	2.0 g
Or L-phenylalanine	1.0 g
Disodium hydrogen phosphate	1.0 g
Sodium chloride	5.0 g
Agar	12.0 g
Distilled water	1.0 L

Adjust the pH to 7.4, distribute and sterilize by autoclaving at 121 °C for 15 minutes.

Allow to solidify in tubes as long slopes.

Principle and interpretation

This test indicates the ability of an organism to deaminate phenylalanine with the production of phenylpyruvic acid, which will react with ferric salts to give a green color. Deamination of phenylalanine and utilization of malonate can be combined in one test.

Procedure

The agar slant of the medium is inoculated with a single colony of the test organism isolated in pure culture of primary plating agar. After incubation at 35 ºC for 18-24 hours, 4-5 drops of the ferric chloride reagent are added directly to the surface of the agar. As the reagent is added, the tube is rotated to dislodge the surface colonies.

Result

Positive test: A green color will develop in fluid and in the slope within one minute.
Negative test: No color change

Positive	Negative
-	*Escherichia coli*

3. β-galactosidase (ONPG) test

Use: To rapidly differentiate delayed lactose-fermenting organisms from lactose-negative organisms.

Ingredients and preparation

Sodium phosphate buffer	0.01M, pH 7.0
O-nitrophenyl-β-D-galactopyranoside (ONPG)	80 mg
Distilled water	15.0 ml

Warm to dissolve crystals, add 5.0 ml phosphate buffer (pH 7. 0) and store in a dark bottle.

Principle

O-nitrophenyl-β-D-galactopyranoside (ONPG) is structurally similar to lactose, except that orthonitrophenyl has been substituted for glucose. On hydrolysis, through the action of the enzyme β-galactosidase, ONPG cleaves into two residues, galactose and o-nitrophenyl. ONPG is a colourless compound; o-nitrophenyl is yellow, providing visual evidence of hydrolysis.

Lactose-fermenting bacteria possess both lactose permease and β-galactosidase, two enzymes required for the production of acid in the lactose fermentation test. The permease is required for the lactose molecule to penetrate the bacterial cell where the β-galactosidase can cleave the galactoside bond, producing glucose and galactose. Non-lactose fermenting bacteria are devoid of both enzymes and are incapable of producing acid from lactose. Some bacterial species appear to be non-lactose fermenters because they lack permease, but do possess β-galactosidase and give a positive ONPG test. So called late lactose fermenters may be delayed in their production of acid from lactose because of sluggish permease activity. In these instances, a positive ONPG test may provide a rapid identification of delayed lactose fermentation.

Procedure

A loopful of bacterial growth is emulsified in 0.5 ml of saline to produce a heavy suspension. One drop of toluene is added to the suspension and vigorously mixed for a few seconds to release the enzyme from the bacterial cells. An equal quantity of buffered ONPG solution is added to the suspension and the mixture is placed in a 37 ºC water bath. When using ONPG tablets, a loopful of bacterial suspension is added directly to the ONPG substrate resulting from adding 1 ml of distilled water to a tablet in a test tube. This suspension is also placed in a 37 ºC water bath.

Result

ONPG positive *Escherichia coli*

Salmonella choleraesuis subsp. *arizonae*

4. Indole

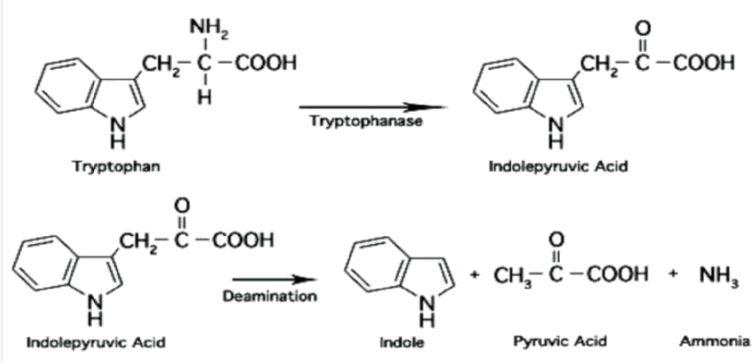
Use: To determine the ability of an organism to split indole from tryptophan.

Ingredients and preparation

The medium is peptone water.

Principle

Indole is generated by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (NH_2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy. Pyridoxal phosphate is required as a coenzyme.



Procedure

Inoculate peptone broth with the test organism and incubate at 35 ºC for 18-24 hours. At the end of this time, add 15 drops of Kovac's reagent down the inner wall of the tube.

Result

Positive test: Reddening of ring formed

Negative test: No red color

Note

1) Kovac's reagent is recommended in preference to Ehrlich's reagent.
2) Tryptone can also be used instead of peptone as an ingredient.
   Positive: *Escherichia coli* Negative: *Klebsiella*

5. Motility test medium (Edwards and Ewing)

Use: This medium is used for checking the motility of organisms. Low agar concentration allows free movement of bacteria.

Ingredients and preparation

Peptone	10.0 g

Beef extract	3.0 g
Sodium chloride	5.0 g
Agar	4.0 g
Distilled water	1.0 L
Final pH at 25°C:	7.4–0.2

Dissolve the ingredients by boiling in 1 L of distilled water. Pour in tubes and autoclave the medium at 121°C for 20 minutes. Cool it in an upright position.

Principle and interpretation

Bacteria are motile by means of flagella. This test is done to determine whether an organism is motile or non-motile.

Procedure

The test isolate is inoculated by stabbing in the center of media in tube with straight wire.

Result

1) Non motile: growth restricted to stab line
2) Motile: Diffused growth ‘‘swarm” extends as a zone of turbidity from the stab line.

Positive	**Negative**
Escherichia coli	*Staphylococcus aureus*
	Klebsiella pneumonia

6. Peptone water

Use: This medium is used as a growth medium for the detection of indole. Testing of indole is important in the identification of enterobacteria. It is also used for the basal medium for sugar fermentation.

Ingredients and preparation

Peptone	10.0 g
Sodium chloride	5.0 g
Distilled water	1.0 L
Final pH at 25 °C:	7.4–0.2

Dissolve by warming. Pour in tubes and autoclave the medium at 121°C for 20 minutes.

7. Simmon’s citrate medium

Use: This medium is used for the ability of the bacteria to utilize citrate as a source of carbon and energy.

Ingredients and preparation

NaCl	5.0 g
$MgSO_4$	0.2 g
$NH_4H_2PO_4$	1.0 g
KH_2 PO_4	1.0 g
Sodium citrate	5.0 g
Distilled water	1.0 L
Bromothymol blue (0.2% aquasolution)	10 ml
Agar (2%)	20 ml

Adjust the pH to 6.8 by adding 1N NaOH (8 ml/L)
Steam for 1 hour and distribute 2.5 ml in each tube and autoclave at 121ºC for 15 minutes. Finally make slopes.

Principle and interpretation
The medium contains citrate, ammonium ions, and other inorganic ions needed for growth. It also contains bromothymol blue, a pH indicator. Bromothymol blue turns blue at a pH of 7.6 or greater. When an organism catabolizes citrate, it produces alkaline waste products, causing the medium to turn blue. Furthermore, only an organism that can utilize citrate will produce visible growth on the citrate slant.

Result
Positive: Blue colored growth
Negative: No growth/ no color change

Positive	**Negative**
Klebsiella	*Escherichia coli*
Salmonella (except *S. typhi* and *S. paratyphi* A)	

8. Sugar fermentation test

Use: It is used to determine the ability of an organism to ferment a specific carbohydrate incorporated in a basal medium and produce acid or acid with visible gas.

Ingredients and preparation

Peptone	10.0 g
Sodium chloride	5.0 g
Distilled water	1.0 L
Final pH at 25°C:	7.4–0.2
Used as basal medium for sugar fermentation	
Andrade’s indicator	1%

To it add different sugar at a concentration of 1% (dextrose, lactose, maltose, sucrose) sterilized by seitz filtration method or autoclave at 10 lbs pressure for 15 minutes.

Principle and result interpretation

Organism ferments the sugar leading to production of acid with or without gas. Acid production is indicated by dark pink color and gas is collected in Durham's tube.

9. Triple sugar iron agar

Use: Triple sugar iron agar is used for the differentiation of enteric pathogens by ability to determine carbohydrate fermentation and hydrogen sulphide production.

Ingredients and preparation

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Glucose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
Ferric citrate	0.3 g
Sodium chloride	5.0 g
Sodium thiosulphate	0.3 g
Agar	12.0 g
Phenol red, 0.2% solution	12.0 ml
Distilled water	1.0 L

Adjust final pH to 7.4. Heat the medium to boil to dissolve constituents completely. Mix well and pour in the tubes. Sterilize at 121ºC for 15 minutes and cool to form slopes with deep butts (3 cm).

Result

Butt color	**Slant color**	**Interpretation**
Yellow	Red	Glucose only fermented
Yellow	Yellow	Glucose fermented, also lactose and/or sucrose
Red	Red	No action on glucose, lactose or sucrose

Bubbles or cracks present: gas production
Black precipitates present: hydrogen sulphide gas production

10. Urease test

Use: To determine the ability of an organism to split urea into two molecules of ammonia by the action of the enzyme, urease, with resulting alkalinity.

Ingredients and preparation

Peptone	1.0 g
Sodium chloride	5.0 g
Di potassium hydrogen phosphate	2.0 g
Phenol red	6.0 ml
Agar	20.0 g
Distilled water	900 ml

Adjust pH at 6.8-6.9 with 1N NaOH

Then add sterile glucose solution 1gm/l. Autoclave at 10 lbs pressure for 20 minutes. Sterilize urea 20 g in 100 ml. by filtration. Distribute 2.5 ml in each sterile tube and slopes are made.

Principle and interpretation

Bacteria, particularly those growing naturally in an environment exposed to urine may decompose urea by enzyme urease. The occurrence of this enzyme can be tested for by growing the organism in the presence of urea and testing the alkali production by means of a suitable pH indicator.

Heavy inoculum of growth is inoculated on the surface of the slants. When urea is utilized ammonia is formed during incubation which makes the medium alkaline showing a pink red color by the change in the phenol red indicator.

Positive	**Negative**
Klebsiella pneumoniae (weakly)	*Escherichia coli*

11. Voges-Proskauer Test

Use: To determine the ability of some organisms to produce a neutral end product, acetylmethyl carbinol (AMC, acetoin) from glucose fermentation.

Ingredients and preparation

Medium is Glucose phosphate (GP) broth

1) α-naphthol 5% color intensifier

α-naphthol	5.0 g
Absolute alcohol	100 ml

2) Potassium hydroxide 40% oxidizing agent

KOH	40.0 g
Distilled water	100 ml

Principle and interpretation

Pyruvic acid the pivotal compound formed in the fermentative degradation of glucose is further metabolized through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathway results in the production of acetoin (acetyl methyl carbinol) a neutral reacting end product in the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and α-naphthol serves as a catalyst to bring out a red complex.

Procedure

Inoculate glucose phosphate broth with a pure culture of test organism. Incubate for 24 hours at 35ºC. Aliquot 1 ml of broth to a clean test tube after incubation period ends. Add 0.6 ml of 5% α-naphthol followed by 0.2 ml of 40% KOH. It is essential that the reagents be added in this order. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Positive	**Negative**
Enterobacter aerogenes	*Escherichia coli*

A positive test is represented by the development of a red color 15 minutes or more after the addition of the reagents.

APPENDIX II

Solvents and Diluents for Preparation of Stock Solutions of Antimicrobial Agents

Antimicrobial Agent	Solvent[a]	Diluent[a]
Amoxicillin, clavulanate, and ticarcillin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Avilamycin	Methanol[b]	Methanol[b]
Cefpodoxime	0.10% (11.9 mmol/L) aqueous sodium bicarbonate	Water
Cephalexin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephalothin[c]	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Chloramphenicol, erythromycin,[d] florfenicol, tylosin,[e] tilmicosin[e]	95% ethanol	Water
Difloxacin	1/2 volume of water, then add NaOH dropwise to dissolve	Water
Enrofloxacin	1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve	Water
Gamithromycin[e]	Phosphate buffer, pH 6.0, 0.2 mol/L with the aid of ultrasonication	Water
Imipenem	Phosphate buffer, pH 7.2, 0.01 mol/L	Phosphate buffer, pH 7.2, 0.01 mol/L
Monensin	Methanol[b]	Methanol[b]
Narasin	Methanol[b]	Methanol[b]
Nitrofurantoin[f]	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 8.0, 0.01 mol/L
Orbifloxacin	1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve	Water
Pradofloxacin	1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve	Water
Rifampin	Methanol[b] (max. concentration = 340 μg/ml)	Water (with stirring)
Sulfonamides	1/2 volume hot water and minimal 1/2 volume hot water and minimal	Water
Tildipirosin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 8.0, 0.1 mol/L
Trimethoprim	0.05 N (0.05 mol/L) lactic[b] or hydrochloric acid,[b] 10% of final volume	Water (may require heat)
Tulathromycin[g]	0.015 M citric acid	Water (can require heat)

Footnotes

a. These solvents and diluents can be further diluted as necessary in water or broth. The products known to be suitable for water solvents and diluents are amikacin, carbenicillin, cefquinome, ciprofloxacin, clindamycin, gentamicin, kanamycin, methicillin, novobiocin, oxacillin, penicillin, pirlimycin, tetracyclines, tiamulin (if hydrogen fumarate), trimethoprim (if lactate), and vancomycin.
b. These compounds are potentially toxic. Consult the safety data sheet before using any of these materials.
c. All other cephalosporins and cephems except ceftiofur and cefquinome not listed above are solubilized (unless otherwise indicated by the manufacturer) in phosphate buffer, pH 6.0, 0.1 mol/L, and further diluted in sterile distilled water. Ceftiofur can be solubilized in water or broth.
d. For glacial acetic acid, use 1/2 volume of water, then add glacial acetic acid dropwise until dissolved, not to exceed 2.5 μl/ml.
e. Solvent can be further diluted as necessary in water or broth.
f. Alternatively, nitrofurantoin, tylosin, and tilmicosin can be dissolved in dimethyl sulfoxide.
g. Tulathromycin requires the use of an equilibrated stock solution. The procedure for producing an equilibrated solution is provided with technical grade powder by the sponsor.

APPENDIX III

Storage and Revival of Bacterial Strains

Bacterial strains may be stored indefinitely at low temperatures (-20 °C and -80 °C) in 15-40% glycerol. For long storage, stock cultures should be stored at -80 °C or in liquid nitrogen. Frozen stock of newly acquired strains should be prepared and archived as soon as possible.

Stock preparation: A freshly grown isolated colony of bacteria should be inoculated into 15 ml culture tube containing 5 ml broth (BHI/NB/LBM/TSB or selective medium containing appropriate antibiotic). The culture should be incubated at 37 °C till late log or stationary phase (usually 5 hours to overnight). After incubation, 225 µl of sterile 80% glycerol should be pipette into a sterile labeled cryovial to archive the isolate at -80 °C. 1.0 ml of the bacterial culture should be added into the cryovial to yield a frozen stock containing glycerol at 15% final concentration. The contents should be mixed well (gentle vortex) and placed at -80 °C. The glycerol stocks should be prepared at least in duplicates or triplicates. If possible, the viability of the stock should be checked after one week.

Recovery of frozen stock: To recover a strain from the -80 °C, glycerol stock should be scraped gently using a sterile toothpick and the scraped ice should be streaked on the appropriate medium e.g. LBM or LBM with antibiotic. Do not thaw the frozen stocks to room temperature because it results in 50% loss in cell viability.
Note: For scraping, it is always better to inoculate first into a recovery broth (Robertson's cooked meat broth or thioglycolate broth) because some organisms may fail to grow directly on solid media from frozen stock.

Recovery of lyophilized cultures: An ampoule should be opened by filing a mark in the middle of the cotton wool plug and applying a red hot glass rod at that site to crack the glass. Air should be allowed to seep into the ampoules sufficiently as hasty opening of ampoule may lead to inward suction of plug upon snapping of the filled end and release of aerosols. The plug should be removed with forceps and discarded into a jar along with broken end of the ampoule. To rehydration and recover the strain, the open end of the tube should be flamed, and 0.3-0.5 ml of NB/TSB should be added using a sterile Pasteur pipette. The contents should be mixed carefully to avoid frothing. A loop-full of broth suspension should be sub-cultured onto appropriate media. The rest of the content should be transferred to a tube containing 1 ml of appropriate broth. Incubate both broth and plate cultures at appropriate conditions

Acknowledgments

Principal Investigator

Dr. Kamini Walia, ICMR, New Delhi

Co-Investigator

Dr. B. R. Shome, ICAR-NIVEDI, Bengaluru

Experts

Dr. (Maj Gen) VC Ohri, ICMR, New Delhi

Dr. Rajesh Bhatia, FAO, New Delhi

Dr. Jyoti Misri, ICAR, New Delhi

Dr. Prakash Halami, CSIR-CFTRI, Mysuru

Dr. Sanjay Bhattacharya, TMC, Kolkata

Dr. V. Balaji, CMC Vellore

Dr. Pallab Ray, PGIMER, Chandigarh

Dr. Samiran Bandyopadhyay, ICAR-IVRI, Kolkata

Dr. Sandeep Ghatak, ICAR RC NEH, Meghalaya

Dr. Tapan Kumar Dutta, CAU Aizawl, Mizoram

Dr. P. X. Antony, RIVER, Puducherry

Dr. J. P. S. Gill, School of Public Health and Zoonoses, GADVASU, Ludhiana

Dr. H. C. Chauhan, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University (SDAU), Gujarat

Dr. S. B. Barbuddhe, ICAR-National Research Centre on Meat, Hyderabad

Prepared and compiled by: Dr. Sonam Vijay, Dr. Monica Sharma; (ICMR, New Delhi)

Reviewed by: Dr. (Maj Gen) VC Ohri, Dr. Kamini Walia, Dr. B. R. Shome

Financial support by: Food and Agricultural Organization (FAO), New Delhi