



Department of AIDS Control

Ministry of Health & Family Welfare, Government of India

Laboratory Manual for Diagnosis of Sexually Transmitted and Reproductive Tract Infections



FOREWORD

Sexually Transmitted Infections (STIs) and Reproductive Tract Infections (RTIs) are diseases of major global concern. About 6% of Indian population is reported to be having STIs. In addition to having high levels of morbidity, they also facilitate transmission of HIV infection. Thus control of STIs goes hand in hand with control of HIV/AIDS.

Countrywide strengthening of laboratories by helping them to adopt uniform standardized protocols is very important not only for case detection and treatment, but also to have reliable epidemiological information which will help in evaluation and monitoring of control efforts. It is also essential to have good referral services between primary level of health facilities and higher levels.

This manual aims to bring in standard testing practices among laboratories that serve health facilities involved in managing STIs and RTIs. While generic procedures such as staining, microscopy and culture have been dealt with in detail, procedures that employ specific manufacturer defined kits have been left to the laboratories to follow the respective protocols. An introduction to quality system essentials and quality control principles has also been included in the manual to sensitize the readers on the importance of quality assurance and quality management system, which is very much the need of the hour.



PREFACE

Sexually Transmitted Infections (STIs) are the most common infectious diseases worldwide, with over 350 million new cases occurring each year, and have far-reaching health, social, and economic consequences. Failure to diagnose and treat STIs at an early stage may result in serious complications and sequelae. Since most of these infections occur in the economically productive age group the issue assumes utmost priority.

STIs are passed from person to person primarily by sexual contact and are classified into varied groups. Some cause mild, acute symptoms and some are life-threatening. They are caused by many different infectious organisms and are treated in different ways. Antimicrobial resistance of several sexually transmitted pathogens is increasing, rendering some regimens ineffective, adding to therapeutic problems.

Apart from being serious diseases in their own right, STIs **enhance the sexual transmission of HIV infection. The presence of an untreated STIs (ulcerative or non-ulcerative) can increase the risk of both acquisition and transmission of HIV by a factor of up to 10.**

It has been estimated that improvement in the management of STIs can reduce the incidence of HIV-1 infection in the general population by about 40%. STIs prevention and treatment are, therefore, an important component in HIV prevention strategy. Correct and early detection of STIs using standardized uniform protocols is essential at all levels in order to institute treatment and control measures.

This manual is written with an intention to bring uniform, detailed testing protocols for STIs and some RTIs at primary, intermediate and central laboratory levels and also to recommend sample transport mechanism and referral network.



ACKNOWLEDGEMENTS



ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AST	Antimicrobial Susceptibility Test
BV	Bacterial Vaginosis
CLIA	Chemiluminescence immunoAssay
DNA	Deoxy Ribonucleic Acid
DSRC	District STI/RTI Center
EIA	Enzyme Immuno Assay
ELISA	Enzyme Linked Immuno Sorbent Assay
EQA	External Quality Assurance
ESR	Erythrocyte Sedimentation Rate
FTA-Abs	Fluorescent Treponema Antibody Absorption Test
GUD	Genital Ulcer Disease
HBV	Hepatitis B Virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HSV	Herpes Simplex Virus
KOH	Potassium Hydroxide
LGV	Lympho Granuloma Venereum
MSM	Men who have Sex with Men
NAAT	Nucleic Acid Amplification Test
PCR	Polymerase Chain Reaction
QA	Quality Assurance
RNA	Ribonucleic Acid
RPR	Rapid Plasma Reagin Test
RSTRRL	Regional STI Training Research and Reference Laboratory
RTI	Reproductive Tract Infection
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SRC	State Reference Center
STI	Sexually Transmitted Infection
TMA	Transcription Mediated Amplification
TPHA	Treponema Pallidum Haemagglutination Test
TV	<i>Trichomonas vaginalis</i>

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A. INTRODUCTION

Sexually transmitted infections (STIs), earlier known as sexually transmitted diseases or venereal diseases, are infections which are predominantly transmitted during sexual contact.

More than 30 bacterial, viral and parasitic pathogens are transmissible sexually and constitute a group of infections referred to as STIs. Most common are Gonorrhoea, Chlamydial infection, Syphilis, Chancroid, Human Immunodeficiency Virus (HIV) infections, Herpes progeneralis (Herpes simplex virus infection), Trichomoniasis, Candidiasis, etc. Although some of the pathogens can be acquired through routes other than sexual transmission, epidemiologically, sexual contact is more important for their transmission from one person to another.

The infected individual acts as a source of infection for other persons. The infection may occur during heterosexual or homosexual activities. STI patients may give a history of multiple sex partners but contact with even a single infected sex partner may be enough to contract infection.

STIs, remain an important focus area for global public health. Public health strategies for STIs control include promotion of safer sexual behaviour and provision of condoms (primary prevention), as well as early and efficacious management of patients with STIs, using either syndromic or etiologic management approaches.

Reproductive Tract Infections (RTIs) include sexually transmitted infections as well as infections of the reproductive tract that are not sexually transmitted. RTIs including STIs and HIV/AIDS are being increasingly recognized as a serious public health problem. RTIs cause suffering both to men and women. RTIs present commonly with vaginal and urethral discharges, pain or burning with urination, itching or irritation of the genitals, sores, blisters or swellings on the genitals and pelvic pain. Adolescents too are vulnerable to RTIs due to their ignorance of risk factors, inadequate accessibility to services and social powerhouses. A number of studies have been initiated to cover the epidemiological, clinical and diagnostic dimensions of RTIs.

1. STI and RTI caused by Bacteria

- a) **Gonorrhoea** is one of the most common STIs and is caused by *Neisseria gonorrhoeae*. Men with gonorrhoea may present with a burning sensation while urinating and a discharge from the urethra, whereas women may present with discharge from the vagina due to cervicitis and lower abdominal pain. Gonorrhoea does not cause vaginitis, however, causes endocervicitis in women. A **discharge** is a yellowish or whitish substance released from the opening of the reproductive tract in both men and women. Most men infected with gonorrhoea have symptoms, but in women gonorrhoea is commonly asymptomatic (i.e. they do not have any symptoms). Women who have gonorrhoea (with or without symptoms) can transmit the bacteria to infants during birth. In newborn babies, gonorrhoea usually presents with eye disease (termed neonatal conjunctivitis / Ophthalmia neonatorum) and can lead to blindness.



- b) **Chlamydia** is also one of the most common STIs, and is caused by bacteria called *Chlamydia trachomatis*. In men it usually presents with discharge from the urethra and in women it presents with cervicitis (inflammation of the neck of the womb or cervix) and lower abdominal pain. The **discharge** is generally less 'sticky' and lighter in colour than for gonorrhoea. Chlamydia, like gonorrhoea, can also be asymptomatic, but in this case in both men and women. There are 15 serovars of which, three of them (L1-L3) cause Lymphogranuloma Venereum (LGV), which starts with a transient genital ulcer and becomes systemic. In addition, pregnant women with chlamydia can also transmit the STI to their babies during childbirth and cause respiratory infections.
- c) **Syphilis** is caused by the spirochete *Treponema pallidum*. Syphilis has four stages: primary, secondary, latent and tertiary syphilis, with different signs and presentations according to the duration since the initial infection. The different stages can be described as follows:
- ▶ **Primary syphilis** is characterised by a painless, indurated ulcer (known as hard/primary chancre) in the genital/oral/anal area resulting from direct sexual contact with a person with syphilis. The chancre has obvious edges, and the lymph nodes in the groin may also appear swollen. Primary syphilis takes 10 to 90 days to develop from initial exposure to the bacterium.
 - ▶ **Secondary syphilis** is characterised by a non-itchy rash over the trunk and the extremities, arising 1 to 6 months after primary syphilis.
 - ▶ **Latent syphilis** is the stage between secondary and tertiary syphilis in which an infected patient shows few or no symptoms.
 - ▶ **Tertiary syphilis** is a rare phenomenon characterised mainly by involvement of meningeovascular, cardiovascular or musculoskeletal systems, granulomatous lesions (gumma). Tertiary syphilis takes 1 to 10 years to develop, but it can take up to 50 years.
- d) **Chancroid** is caused by a bacteria termed *Haemophilus ducreyi* and in the majority of cases it presents with non-indurated and painful ulcers (soft chancre) unlike primary syphilitic chancre (hard chancre), and sores in the genital area (particularly in the foreskin of the penis). Many patients also develop a bubo, an painful enlargement of the lymph nodes on one side of the groin that exudate.
- e) **Granuloma inguinale** (Donovanosis) is caused by an infection with a bacteria called *Klebsiella granulomatis*. It presents initially with small lesions in areas surrounding the anus and/or genitals, which are difficult to differentiate from chancroid, but then turn into ulcerative lesions and lead to painless raised solid bumps in both sides of the groin area.
- f) **Bacterial vaginosis** is associated with overgrowth of certain organisms (*Gardnerella vaginalis*, *Mobiluncus mulieri*, *M. curtissi*, *Bacteroides bivius* and Anaerobic cocci) which disrupt the normal bacterial flora of the vagina. BV is an endogenous infection which is associated with increased risk behavior. Patients present with foul smelling, grey homogenous and thin vaginal discharge.
- g) ***Mycoplasma hominis* and *Ureaplasma urealyticum*** cause urethritis, cervicitis and pelvic inflammatory disease.

2. STIs caused by Viruses

HIV/AIDS is caused by Human Immunodeficiency Virus.

Lab diagnosis of HIV is dealt extensively in a separate manual (Guidelines on HIV Testing, Department of AIDS Control).

- a. **Herpes genitalis** is the most common STI caused by a viral infection. The pathogen responsible for genital herpes is Herpes simplex virus type 2 (HSV-2, though HSV-1 also can). Genital herpes usually presents with blisters, when they break it leads to painful sores and ulcers in the outer surface of the genitals and in areas surrounding the anus.
- b. **Genital wart** is a viral STI caused by Human Papilloma Virus (HPV) and commonly presents with small fleshy growths of skin on the genital area or around the anus. HPV has also been shown to be the causative agent of cervical cancer in women. However, the types of HPV that cause genital warts are not the same as the types that can cause cancer (HPV-16, 18) which is usually asymptomatic for years.
- c. **Hepatitis B virus (HBV) and Hepatitis C virus (HCV)** are primarily blood borne pathogens transmitted through blood, blood products, needles and body fluids. Sexual mode of transmission of Hepatitis C is not proven, though its prevalence is high among IV drug users and MSMs.

3. STI caused by a protozoan

Trichomoniasis is a STI caused by a protozoan, *Trichomonas vaginalis* that is usually found in vaginal and urethral tissues. It presents in women with profuse and frothy vaginal discharge. Although this condition is most often treated in women, men can also be infected but often show no symptoms.

4. RTI caused by fungus

Vaginal candidiasis is a vaginal infection caused most often by a fungus termed *Candida albicans* and nowadays increasingly by other candida species. The main symptoms of candidiasis in women are a curd-like vaginal discharge, vaginal itching and sometimes a burning sensation. A patient may be infected with a single organism or may have multiple organisms at the same time.

Based on the types of lesion and the presenting symptoms, STIs can be grouped as those with a) ulcers b) discharge c) pain and swelling and d) others. This classification is useful to facilitate sample collection and syndromic treatment. *

Syndrome	Signs and symptoms	Common Cause	Specimen to be collected
Vaginal discharge	Unusual vaginal discharge, vaginal itching, dysuria (pain on urination) and dyspareunia (pain during sexual intercourse)	Trichomoniasis, Candidiasis, Bacterial vaginosis, Gonorrhoea, Chlamydial infection	Vaginal/cervical (discharge) swabs

Syndrome	Signs and symptoms	Common Cause	Specimen to be collected
Urethral discharge	Urethral discharge, dysuria, frequent urination	Gonorrhoea, Chlamydia	Urethral Swabs, urine
Genital ulcer	Ulcer/sore	Syphilis, Chancroid, Genital herpes, Granuloma inguinale, Lympho Granuloma Venereum (LGV)	Swabs/aspirates/serum
Lower abdominal pain	Vaginal discharge, fever, lower abdominal pain and tenderness	Gonorrhoea, Chlamydia mixed anaerobes	cervical/vaginal/swabs/aspirate
Painful scrotal swelling	Pain and swelling of the scrotum	Gonorrhoea, Chlamydia	Aspirate
Inguinal bubo	Painful, enlarged lymph nodes on the groin	LGV, Chancroid	Aspirate

* Syndromic management is based on the identification of consistent groups of symptoms and easily recognized signs (syndromes), and the provision of treatment that will deal with the majority or most serious organisms responsible for producing a syndrome.

B. STRATIFICATION OF LABORATORIES

Recognizing the need to provide prompt diagnosis for initiation of treatment and also keeping in mind that many confirmatory tests require specialised equipment, expertise or environment, laboratories have been classified into three levels. A referral network system from periphery to Intermediate and Central laboratory is available.

1. **Peripheral/Primary level Laboratory:** Located at STI clinics, Primary Health Centres, dispensaries MCH/FP clinics, Taluk hospitals, some district hospitals etc., these laboratories have limited facilities in terms of equipment and trained manpower. **A light microscope (bright field microscope) is made available** for the examination of wet mount preparation and stained smears. A dark field microscope may be available in areas where syphilis is fairly common in the general population. Point of care tests are also done here.
2. **Intermediate Laboratory/State Reference Laboratory:** These laboratories are located at identified Medical Colleges and many district hospitals. In addition to primary level facilities, these laboratories have facilities to perform some cultures, identification, antibiotic sensitivity testing and serology tests. They are responsible for quality assurance and training of Primary laboratories assigned to them.
3. **Central/Regional Reference Laboratory:** These laboratories are well- equipped with state of the art equipment and highly trained staff to perform more sophisticated confirmatory tests apart from the tests suggested at primary and intermediate laboratories. They undertake diagnosis, surveillance and research in sexually transmitted infections in their areas. These laboratories also are expected to ensure quality of tests performed in the intermediate laboratories/state reference laboratories linked to them, to train the laboratory staff and participate in evaluations /studies organized by the apex laboratory. Regional STI referral laboratories, well-equipped medical colleges and selected BioMedical Research institutes constitute central laboratories.
4. **Apex reference Laboratory:** In addition to functioning as the Regional reference laboratory for the state reference labs attached to it, the apex laboratory will organize and conduct External Quality Assurance Program and training programs in STIs. It will also serve as a collaborating Centre with WHO or any other international organization for surveillance in STIs and evaluation of new diagnostic tests/algorithms.

Taking into consideration the resources available in the country, the tests recommended at the different levels of the health care system are shown in the following Table.

Aetiological Agent/ Condition	Diagnostic Test	Level of laboratory			
		Primary	State reference	Regional reference	Apex
<i>Neisseria gonorrhoeae</i>	Smear – Gram stain	+	+	+	+
	Culture	-	+	+	+
	Identification of organism	-	+	+	+
	β-lactamase test	-	±	+	+
	Antimicrobial susceptibility test		±	+	+
	Nucleic Acid Amplification	-	±	+	+
<i>Chlamydia trachomatis</i>	Antigen detection 1)Direct fluorescent antibody technique (DFA)	-	-	+	+
	2)ELISA	-	-	+	+
	Culture	-	-	±	+
	Nucleic Acid Amplification	-	-	+	+
<i>Treponema pallidum</i>	Dark field Microscopy	-	+	+	+
	RPR Test Qualitative	+	+	+	+
	RPR Test Quantitative	-	+	+	+
	VDRL (Qualitative)	-	+	+	+
	VDRL (Quantitative)	-	+	+	+
	TPHA	-	+	+	+
	FTA-Abs test	-	-	±	+
	Molecular identification	-	-	±	+

Aetiological Agent/ Condition	Diagnostic Test	Level of laboratory			
		Primary	State reference	Regional reference	Apex
<i>Haemophilus ducreyi</i>	Smear	+	+	+	+
	Culture	-	±	+	+
	β-lactamase test	-	±	+	+
	Antimicrobial susceptibility test	-	-	+	+
<i>Klebsiella granulomatis</i>	Tissue smear - Giemsa stain	-	±	+	+
Herpes Simplex Virus-1&2	Smear - Tzanck Giemsa stain	-	±	+	+
	Antigen detection 1) Immuno-fluorescent staining	-	±	+	+
	2)ELISA	-	±	+	+
	Antibody detection Anti- HSV IgM (type specific glycoprotein) G[gG]	-	±	+	+
	Culture	-	-	±	+
	Nucleic Acid Amplification	-	-	+	+
<i>Candida</i> spp.	Potassium hydroxide Wet mount	+	+	+	+
	Smear - Gram stain	+	+	+	+
	Culture	-	+	+	+
	Speciation & AST	-	±	+	+
<i>Trichomonas vaginalis</i>	Saline wet mount	+	+	+	+
	Staining - Giemsa stain	±	+	+	+
	Acridine Orange staining	-	-	±	+
	Culture	-	±	+	+

Aetiological Agent/ Condition	Diagnostic Test	Level of laboratory			
		Primary	State reference	Regional reference	Apex
Bacterial vaginosis	Saline wet mount	+	+	+	+
	Smear - Gram stain	+	+	+	+
	pH test, KOH test	+	+	+	+
Human Papilloma Viruses	Nucleic Acid Amplification	-	-	+	+
Hepatitis B virus	Immunochromatography HBs Ag detection	+	+	+	+
	ELISA for HBs Ag detection	-	±	+	+
	Nucleic Acid Amplification Tests	-	±	±	+
Hepatitis C virus	Anti-HCV antibody detection	-	±	+	+
	Nucleic Acid Amplification Tests	-	±	±	+

C. COLLECTION AND TRANSPORT OF SPECIMENS

Collection of an adequate specimen from the proper site from the right patient with symptoms is essential for correct diagnosis (**Right patient, right test and right sample!**). The site for collection of the specimen will depend on the clinical symptoms. It is essential to follow Standard precautions at all times during specimen collection, storage, testing, transportation and disposal of bio-hazardous wastes. Standard precautions are meant to reduce the risk of transmission of blood borne and other pathogens from both recognized and unrecognized sources.

The anogenital specimen in males can be collected by the medical officer himself or by the technician. In females the specimen should, preferably, be collected by a lady medical officer, a nurse or a lady health worker only.

Specimens that are common to most of the infections:

1. Swabs-e.g. urethral, cervical, vaginal, anogenital, oropharyngeal ulcers etc.
2. Aspirates-e.g. sores or buboes
3. Body fluids- e.g. urine
4. Blood for serology-whole blood or serum
5. Blood /Urine/Ulcer Swabs for Nucleic acid testing
6. Sometimes culture plates may be directly inoculated at site of collection and transported
7. Tissue/Crush smear

Materials required for collection of specimens:

1. Sterile gloves
2. Sterile cotton wool swab sticks/ Serum albumin coated swabs
3. Vaginal speculum (for females)
4. Proctoscope/anoscope
5. Microscope glass slides
6. Normal saline
7. Bacteriological loop
8. Spirit lamp
9. Glass marking pencil / marker
10. Coverslips
11. Match-box
12. Tourniquet
13. Blood/specimen collection tubes
14. Syringe and needle (Evacuated system, if available)
15. Alcohol swabs, cotton and sterile gauze as required

Preparation of albumin coated swabs:

Dip cotton swabs in 20% bovine serum albumin (BSA) solution. Dry at 37° C and then sterilize by autoclaving. Serum (horse or sheep) can replace BSA.

Method of collection of specimens:

Note: All specimen collections for culture should be performed wearing sterile gloves.

Specimen Type	Method of Collection
Urethral swab	<p>Should be done after atleast one hour of voiding urine. Express urethral exudates when patients have discharge, collect with a sterile swab.</p> <p>If there is no discharge, compress the meatus vertically to open the distal urethra and insert a thin, water-moistened swab (calcium alginate or Dacron) with flexible wire slowly (3 cm to 4 cm in males or 1 cm to 2 cm in females), rotate slowly and withdraw gently. Do not use calcium alginate swabs for viral cultures.</p>
Epididymis	Use a needle and syringe to aspirate material from the epididymis and collect in a tube.
Urine	<p>Ask patients to collect only the first 10 mL to 15 mL of urine in a sterile container. Patients should not have voided for at least 2 hrs before specimen collection to increase the chance of detecting the organism. For nucleic acid testing, collect 20ml of first-voided urine.</p> <p>Do not collect urine routinely for culture of <i>N.gonorrhoeae</i></p>
Cervical swab	<p>Insert a speculum into the vagina to view the cervix. Wipe the cervix clean of vaginal secretion and mucus.</p> <p>Insert a swab 1 cm to 3 cm into the endocervical canal and rotate for 10 s to 30 s to allow absorption of exudates</p> <p>In cases of suspected coinfections of <i>N. gonorrhoeae</i> and <i>Chlamydia trachomatis</i>, the cervical specimen for <i>N. gonorrhoeae</i> detection should be taken before the specimen for <i>C. trachomatis</i>, because <i>N. gonorrhoeae</i> is present in the mucus from the endo-cervix and <i>C. trachomatis</i> is present in the cervical epithelial cells. A small brush on a wire (cytobrush) is used to collect specimens in females in cases of <i>C.trachomatis</i> infections.</p>

Specimen Type	Method of Collection
Vaginal swab	Collect pooled vaginal secretions, if present. Vaginal wash specimens* are most preferred and acceptable from prepubertal girls. If not possible, rub a sterile cotton swab against the posterior vaginal wall and allow the swab to absorb the specimen.
Rectal swab	Specimens may be obtained blindly or, preferably, through an anoscope. Insert a swab 2 cm to 3 cm into the anal canal. Avoiding fecal material, rotate to sample crypts just inside the anal ring; allow the swab to absorb specimen for 10 s.
Oropharyngeal	Depress the tongue gently with tongue depressor Rub sterile swabs over the posterior pharynx and tonsillar crypts, or obtain nasopharyngeal aspirate from infants. Avoid touching other surfaces of mouth.

Note: Do not use lubricant or antiseptics during sample collection.

*Sterile dry swabs are used to collect genital secretions from the endocervix, the vaginal wall, and the vaginal fornix. Apply swabs gently to the mucosal walls, and apply slight pressure by partly rotating the swabs without any mucosal trauma. Store all the swab samples immediately at -80°C until use. Following genital swab sampling, whole vaginal secretions are collected by a standardized 60-s vaginal washing with 3 ml of phosphate saline buffer (pH 7.2).

Collection of blood samples for serology and nucleic acid testing

Materials needed:

Collect all the materials needed for the procedure and place it within safe and easy reach on a tray or trolley, ensuring that all the items are clearly visible.

- ▶ Sterile glass or plastic tubes for serology and EDTA tubes for nucleic acid testing; vacuum extracted tubes are preferable.
- ▶ Syringe and needles
- ▶ Well-fitting, non-sterile gloves
- ▶ A tourniquet
- ▶ Alcohol hand rub
- ▶ 70% alcohol swabs for skin disinfection
- ▶ Gauze or cotton-wool ball to be applied over puncture site
- ▶ Laboratory specimen labels
- ▶ Writing pen
- ▶ Laboratory forms
- ▶ Leak-proof transportation bags and containers;
- ▶ A puncture-resistant sharps container.

Steps:

- ▶ Identify and prepare the patient.
- ▶ Select the site-extend patient's arm and inspect the antecubital fossa or forearm.
- ▶ Locate a vein of a good size that is visible, straight and clear.
- ▶ The vein should be visible without applying the tourniquet.
- ▶ Apply the tourniquet about 4–5 finger widths above the venipuncture site and re-examine.
- ▶ Disinfect the entry site with alcohol.
- ▶ Draw blood.
- ▶ Fill in the tubes.
- ▶ Discard the needle in puncture-proof container; syringes in red coloured bin.
- ▶ Allow the blood to clot fully for 20-25 mins
- ▶ Centrifuge at 3000 rpm for 10-15 mins
- ▶ Pipette the supernatant serum into another sterile tube; label it.
- ▶ Use this for serological tests and if required, to transport to reference laboratory.

Transport of specimens

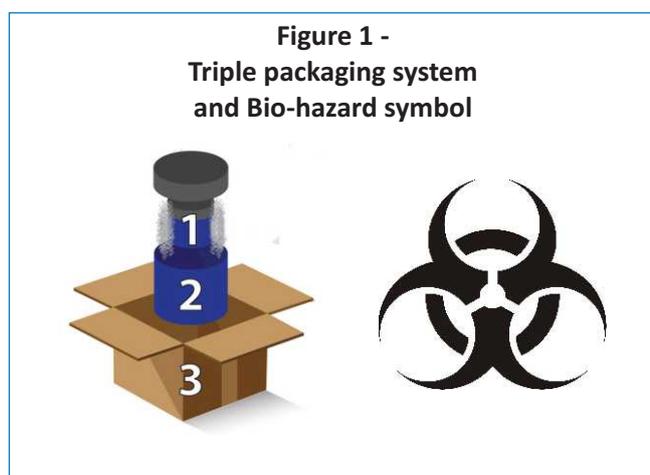
Specimens and associated materials must be packaged and transported in a suitable manner, to:

- ▶ protect the safety of everyone required to handle the specimens and package
- ▶ ensure that the material is maintained under suitable conditions and is stable to perform the required test.

Packing of specimens:

The system consists of three layers as follows

1. **Primary receptacle.** It is a labeled primary watertight, leak-proof receptacle containing the specimen. The receptacle is wrapped in enough absorbent material to absorb all fluid in case of breakage.
2. **Secondary receptacle.** It is a second durable, watertight, leak-proof receptacle to enclose and protect the primary receptacle(s). Several wrapped primary receptacles may be placed in one secondary receptacle. Sufficient additional absorbent material must be used to cushion multiple primary receptacles. Specimen data forms, letters and other types of information that identify or describe the specimen test and also identify the shipper and receiver should be taped to the outside of the secondary receptacle, preferably in a zip pouch.
3. **Outer shipping package.** The secondary receptacle is placed in an outer shipping package which protects it and its



contents from outside influences such as physical damage and water while in transit. The tertiary container must bear the mailing label which identifies the shipper and receiver along with biohazard sign.

4. Ice or dry ice required to maintain temperature should be placed in the secondary receptacle.

Ziploc plastic bags may also be used as leak-proof containers if suitable boxes are not available. Packed specimens should be sent to the nearest referral laboratory for further tests.

Sample	Temperature for transport
Slides for microscopy	Ambient
Swabs for viral culture	2-8°C*
Serum for Immunoassays	2-8°C
Blood for nucleic acid testing	2-8°C

* VTM (Viral Transportation Medium) is recommended for transportation of samples for viral culture at 2 to 8 °C. Samples should be transported within 48 hours.

D. LABORATORY PROCEDURES

1. GONORRHOEA

Gonorrhoea is caused by aerobic gram-negative diplococci, *Neisseria gonorrhoeae*. Demonstration of intracellular gonococci in Gram stained smears made from urethral discharges have very good sensitivity and specificity at primary care level. In cases with cervical oro-pharyngeal and rectal discharges, culture will have to be performed as microscopy has very poor sensitivity and specificity.

1.1 Sampling requirements:

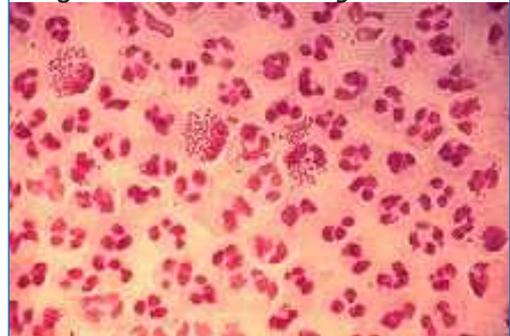
Test	Technique	Specimen
Microscopy	Gram staining	Swab from discharge
Culture of <i>N.gonorrhoeae</i>	Aerobic culture, antibiotic sensitivity	Swabs from Urethra, endocervix, rectum,pharynx
DNA probe	Nucleic acid hybridisation	Urethral, endocervical swab
PCR	Nucleic acid amplification	Urethral, endocervical, rectal, pharyngeal swabs, urine.

1.2 Microscopy

Purpose of microscopy is to stain the smears by Grams method and look for pus cells, intracellular Gram-negative diplococci suggestive of gonococci.

1. With one swab prepare a smear on a clean grease free slide (Annexure-I)
2. Allow the smear to dry in the air.
3. Heat fix the smear (Annexure-I)
4. Stain with Gram stain (Annexure-I).
5. Examine the slide using light microscope. First focus under 10x objective and then put a drop of liquid paraffin (immersion oil) on the smear and examine under 100x objective. Push the condenser up and open the iris diaphragm so that maximum light passes through the slide.

Figure 2 - Gram Stain of *N. gonorrhoeae*

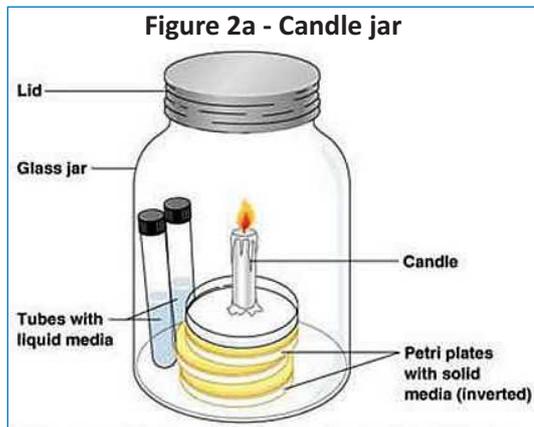


Reading: Examine the smear for epithelial cells, polymorphonuclear leucocytes (pus cells), organisms and their location- whether extracellular or intracellular. The gonococci are intracellular, bean shaped and are usually arranged in pairs, 0.8µm x 0.6µm in size. They are

Gram negative in reaction and are stained pink along with the nuclei and protoplasm of pus cells. Examine the slide for at least 2 minutes before declaring them negative.

1.3 Transport of samples

1. Ideally, the specimens should be inoculated onto culture medium immediately after collection to preserve the viability of gonococci for isolation. If the inoculated media are being transported to the next-level laboratory, the plates should be held at room temperature for no more than 5 hours in a CO₂ enriched atmosphere using a candle jar or a commercial CO₂ generating system. If long-distance shipping is required, the specimens should be inoculated onto media contained in a CO₂ generating system, incubated for 18 hours to 24 hours and should have visible growth on the plate before shipping.
2. If direct inoculation is not possible, transport one swab to the laboratory.
3. Delays in the transport may cause the organisms to die. Therefore, send the swab in a transport medium like Stuart medium or Amies medium (Annexure-II). These can be left at room temperature. The isolation rate after transport of specimens in these at room temperature (25^o C) is approximately 100% within 12 hours, and more than 90% within 24 hours, although the number of colonies decreases markedly. A selective growth and transport medium such as the InTray™ GC (gonorrhoea)-Biomed Diagnostics, or Transgrow or Jembec can also be used, if available. In these bacteria survive for 2 days. The InTray™ GC system is equipped with an integrated CO₂ tablet contained in a sealed inner chamber to prevent degradation during storage. Once the CO₂ chamber is punctured and the InTray™ sealed, the tablet generates the required anaerobic atmosphere of CO₂ gas (approximately 7%).
4. Transport of samples should be done in Triple-packing method as described in Section C (Collection and Transport of Specimens)



1.4 Culture for *N.gonorrhoeae*

Material required:

1. Media plates – Chocolate agar (Columbia agar base plus sheep/horse blood) and Saponin lysed blood agar plus VCNT (Vancomycin, Colistin, Nystatin, Trimethoprim) inhibitors. Alternatively, GC agar base may be used with haemoglobin and VCNT. Basically, two plates should be used; one with inhibitors and one without it. (Annexure-II).
2. Desiccator (Candle jar) or a CO₂ incubator
3. Candle (white wax)

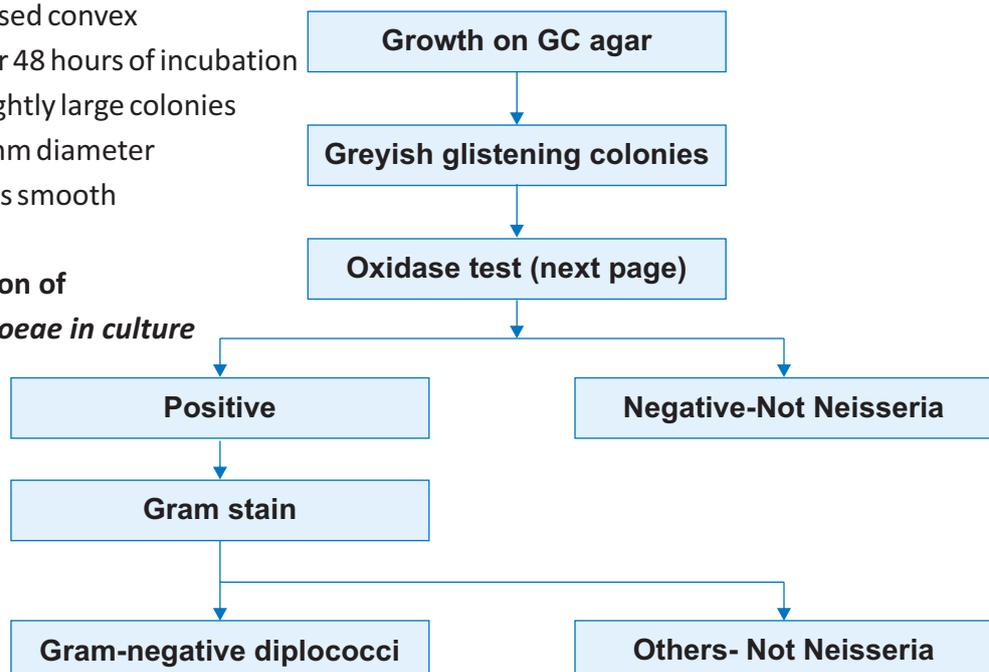
4. Moist cotton/tissue roll.
5. Incubator set at a temperature of 35°C - 37°C.
6. Inoculating loop
7. Glass marking pencil / permanent marker
8. Sterile Distilled water

Method:

1. Inoculate the specimen on the culture plate.
2. Roll the swab on ¼th of the plate surface or 1 cm. circular area of the agar plate.
3. Spread the inoculated material on the whole plate in a “Z” pattern with a sterile nichrome loop.
4. Open the candle jar and keep the moist cotton ball/ wet paper towels inside the jar to provide >70% humidity.
5. Light a candle and keep it upright in the lower part of the candle jar to provide 5% CO₂.
6. Keep the inoculated media plates in the upper part of candle jar.
7. Close the lid of the jar.
8. After the candle stops burning keep the whole candle jar with the culture plates in the incubator.
9. Examine the plates after 18-24 hours. If no growth, then incubate the plates for another 24 hours.
10. Examine the plates after 48 hours. Read the colonies. If no growth, report as such.
11. Reading of plates:

- a. After 24 hours of incubation
 - Small colonies- pin point
 - 0.5 to 1mm in diameter
 - Grey to white in colour, smooth
 - translucent
 - raised convex
- b. After 48 hours of incubation
 - slightly large colonies
 - 3mm diameter
 - less smooth

1.5 Identification of *N. gonorrhoeae* in culture



Perform oxidase, superoxol and rapid/ conventional test for carbohydrate utilization to confirm the identity of *N.gonorrhoeae* and follow with antibiotic sensitivity testing (see below)

1.5.1 Oxidase test: *N. gonorrhoeae* is oxidase positive.

Material required:

- i) Oxidase reagent (tetra methyl para- phenylenediamine dihydrochloride).
- ii) Distilled water.
- iii) Filter paper strips.
- iv) Glass slides / wooden applicator stick

Method I:

- i) Prepare a 1% fresh solution of oxidase reagent by dissolving 10 mg of reagent in 1 ml of distilled water.
- ii) Put a drop of the reagent on the colony to be tested.
- iii) Immediate development of a purple colour indicates a positive test.

Method II:

- i) Moisten a filter paper strip with 2-3 drops of oxidase reagent.
- ii) Pick one colony with the wooden applicator stick or tip of glass slide or tooth pick or platinum loop and rub on filter paper.

Method III:

- i) Cut Whatman filter paper into small strips.
- ii) Pour 1% fresh solution of oxidase reagent on to the strips in a big petri dish.
- iii) Dry them in hot air oven.
- iv) Store the strips in a brown bottle in refrigerator.

Reading:

- i) Dark deep purple colour in 5–10 sec – positive test.
- ii) Deep purple colour in 10–60 sec. – Delayed positive test.
- iii) No colour or colour development after 60 sec. – negative test.

Positive control: *N. gonorrhoeae* WHO C.

Negative control: *Escherichia coli* ATCC 25922.

1.5.2 Superoxol test:

Slide method:

- i) Place a drop of 30% w/v hydrogen peroxide in the centre of a clean glass slide.
- ii) Pick up a few colonies of the culture to be tested from the plate with a wooden

applicator stick or tip of glass slide or tooth pick and emulsify directly in the drop of hydrogen peroxide.

- iii) Immediate production of bubbles (within 1 to 2 seconds) is defined as a positive result. A negative reaction is defined by weak or delayed bubbling after 3 seconds.

Plate method:

Perform by placing one drop of 30% w/v hydrogen peroxide solution on chocolate agar. An immediate and abundant production of bubbles indicates a positive test. Delayed or weak bubbling indicates a negative test.

N. gonorrhoeae (100%) and some other commensal *Neisseria* give a positive reaction. More than 98% of *N. meningitidis* are negative in this test. A negative test means that the isolate is not a gonococcus.

1.6 Identification of Neisseria based on sugar utilization

Rapid carbohydrate utilization test (RCUT):

Carbohydrate utilization tests are the most frequently used methods. The rapid carbohydrate utilization tests, which are non-growth dependent and combine ease of performance, rapidity and reliability with low cost, are recommended.

Acidometric test for the presence of beta-lactamase, may be conveniently performed along with RCUT. β -lactamase, an extracellular enzyme produced by many strains of bacteria, specifically hydrolyses the amide bond in the β -lactam ring of penicillin analogues, rendering the antibiotic inactive. Penicillinoic acid is formed with a resulting colour change. A test for β -lactamase from *N. gonorrhoeae* can be detected by the change in colour of phenol red pH indicator (red to yellow) when penicillin converts to penicillinoic acid.

Ampicillin, is more stable and more sensitive to TEM β -lactamase, and is used instead of penicillin G for the test. The test can thus be used both for identification and testing for β -lactamase production.

Materials required:

1. Buffered salt indicator solution
2. Growth medium, e.g., non-selective chocolate agar plates
3. Carbohydrate solutions
 - 10% glucose (G)
 - 10% lactose (L)
 - 10% sucrose (S)
 - 10% maltose (M)
4. Ampicillin solution for β -lactamase test

Procedure:

A pure culture for the identification of suspected *N. gonorrhoeae* is obtained by sub culturing a single colony. Emulsify two full 3 mm loopfuls of the isolate from a pure (24 hr) culture into a tube containing 1.5 ml buffered balanced salt indicator solution (BSS) and mixing well with a Pasteur pipette to obtain approximately 10^9 organisms per ml.

Take a microtitre plate and mark its 6 wells as C (control), G (Glucose), L (Lactose), M (Maltose), S (Sucrose) and P'ase (penicillinase). Add 25 μ l of 10% sterile sugar solution to G, L, M, S wells and 25 μ l of ampicillin solution (200 mg/mL) to P'ase well. The first well without any sugar will serve as a control. Add 100 μ l (4 drops) of bacterial suspension to each of the six wells.

Read after 2 to 4 hours of incubation at 35°C - 37°C in air (not in CO₂). It is recommended that the β -lactamase reaction is examined again after 24 hours as slow β -lactamase reactions occur with occasional strains.

The following control strains are set up simultaneously with each test run.

Neisseria gonorrhoeae WHO E or WHO O: β -lactamase POSITIVE

Neisseria gonorrhoeae WHO C or WHO K: β -lactamase NEGATIVE

Note:

1. If *N.gonorrhoeae* is suspected and a doubtful result is obtained with the rapid carbohydrate utilization test, check the purity of the culture and send it to the central laboratory for confirmation.
2. The RCUT should not be performed in glass tubes.

Results:

The results of carbohydrate utilization and β -lactamase production are recorded as follows:

Control tube/well = Red.

Yellow colour = Positive reaction

Orange Red colour = Negative reaction

N.gonorrhoeae utilizes glucose only but not maltose and lactose, so only the tube containing glucose should have a colour change.

Interpretation of sugar utilization test

Glucose	Maltose	Lactose	Sucrose	Identification
+	-	-	-	<i>N.gonorrhoeae</i>
+	+	-	-	<i>N.meningitidis</i>
+	+	+	-	<i>N.lactamica</i>
-	-	-	-	Refer to reference laboratory for further confirmation

1.7 β -lactamase test

Helps in detecting plasmid mediated resistance of *N. gonorrhoeae* to penicillin. Apart from the use of ampicillin in conjunction with RCUT to detect β -lactamase, other tests are available.

These include:

1. Chromogenic cephalosporin test
2. Acidometric Method
3. Paper iodometric method

▶ **Chromogenic cephalosporin test:**

This test is recognized as the “gold standard”.

Material required:

- i) Chromogenic cephalosporin (Nitrocefin containing disk or freeze dried powder BBL/Oxoid).
- ii) Dilution buffer provided with the powder.

Method:

a) Disk method:

- i) Wet a nitrocefin disk with a drop of distilled water.
- ii) Pick 5 colonies with sterilized platinum loop and rub on the disk

Reading:

Red colour within 1 minute – positive test.

Original yellow colour – negative test.

b) Slide Method:

- i) Prepare nitrocefin solution by mixing freeze dried powder with the dilution buffer.
- ii) Put one drop of nitrocefin solution on a piece of filter paper over a slide.
- iii) Pick up 10-20 colonies of *N. gonorrhoeae* with a sterile platinum loop.
- iv) Rub the colonies with the reagent on the filter paper.

Reading:

Red colour in 30 seconds – Positive test.

Yellow colour – Negative test.

1.8 Antibiotic sensitivity testing

All isolates of *N. gonorrhoeae* should be tested for their susceptibility to clinically important antimicrobial agents. Testing for antimicrobial resistance in *N. gonorrhoeae* is an integral part of a routine STI laboratory programme and monitoring resistance by surveillance is important for disease control.

Patient Populations

- ▶ STI patients (individuals presenting for treatment with signs and symptoms)
- ▶ Screening/case finding (individuals who are mostly asymptomatic for STI, about are tested for gonorrhoea as part of an early detection programme)

Antibiotics to be tested

The following antimicrobial agents should be tested in *N.gonorrhoeae* isolates. The antibiotics in the 'core' group are those currently recommended by the WHO.

Antibiotics in the 'additional' group include those that are used for the treatment of gonorrhoea in some parts of the world.

Core Group

1. Penicillins used as the representative antibiotic. Results apply to ampicillin and amoxycillin.
2. Quinolones ciprofloxacin is currently recommended for testing as the representative quinolone. Results of ciprofloxacin testing may be applied to other quinolone agents.
3. Ceftriaxone used as a representative of injectable third generation cephalosporins.
4. Cefixime used as a representative of oral third generation cephalosporins.
5. Tetracyclines used only to detect strains with high-level plasmid mediated resistance to tetracycline-referred to as TRNG strains.
6. Spectinomycin useful against penicillin and ceftriaxone resistant isolates.
7. Azithromycin recommended as part of a dual treatment strategy.

Additional group

Other cephalosporins (cefepodoxime), aminoglycosides.

Techniques for antimicrobial susceptibility testing of *N. gonorrhoeae*

The disc diffusion technique is widely employed as the initial susceptibility test. In addition, MIC can be determined using agar dilution method or E-test.

1.8.1 Disk-Diffusion method

The disk diffusion method of susceptibility testing (also known as the Kirby-Bauer (KB) method) has been standardized primarily for testing of rapidly growing aerobic bacteria. To perform the test, filter paper disks impregnated with a specific amount of antimicrobial agent are applied to the surface of an agar medium that has been inoculated with a known amount of the test organism. The drug in the disk diffuses through the agar. As the distance from the disk increases, the concentration of the antimicrobial agent decreases creating a gradient of drug concentrations in the agar medium.

Concomitant with diffusion of the drug, the bacteria that were inoculated and that are not inhibited by the concentration of the antimicrobial agent continue to multiply until a lawn of growth is visible. In areas where the concentration of drug is inhibitory, no growth occurs, forming a zone of inhibition around each disk.

1.8.1.1 Clinical Laboratory Standard Institute (CLSI) method:

It was earlier known as National Committee for Clinical Laboratory Standards (NCCLS) method. Quality Control strain to be used: *N.gonorrhoeae* ATCC 49226 and WHO F to P panel of reference strains.

Medium: GC agar base with 1% defined growth supplement(1.1g L-cysteine,0.03g guanine HCl,3mg thiamine HCl,13mg para-aminobenzoic acid,0.01g B₁₂,0.1g cocarboxylase,0.25g nicotinamide adenine dinucleotide,1g adenine,10g L-glutamine,100g glucose, 0.02 g ferric nitrate) or Chocolate agar

Inoculum: pick up few colonies from an overnight (20 -24 hrs) chocolate agar plate; make a direct suspension equivalent of 0.5 MacFarland standard; prepare in Mueller Hinton Broth or 0.9% phosphate buffered saline, pH7.0.

Materials

- Chocolate Agar with growth supplement
- Phosphate buffered saline (Annexure-II)
- Sterile swabs

Filter paper discs with the antibiotic as, follows:

Disk	Concentration of antibiotic
Penicillin	10 IU.
Tetracycline	30 µg.
Ceftriaxone	30 µg.
Spectinomycin	100 µg.
Ciprofloxacin	5 µg.
Cefixime	5 µg.
Azithromycin	15µg

Procedure

1. Allow disks to come to room temperature before opening the container.
2. Prepare a suspension of the test organism in sterile saline equivalent to a 0.5 McFarland standard using isolated colonies.
3. Using a sterile cotton swab, inoculate the organism on agar
4. Using forceps or a disk dispenser, apply the appropriate antimicrobial disks onto the agar. Place the disks with an equal distance apart from each other and put not more than 6 disks on a 90 mm diameter plate.
5. Incubate plates at 35°C for 18-24 hrs.

- If the organism is sensitive, there is a clear zone of inhibition around the disc. If the organism is not sensitive to the antibiotic there is no clear zone around the disk.

Measure the diameter of the clear zone by placing the ruler on the inoculated surface of the petridish (The side on which the code word for the antibiotic is written).The size of the zone indicates the sensitivity of gonococcus (resistant, intermediate sensitive or sensitive).

Read the sensitivity as follows:

Drug	Zone size in mm		
	Resistant	Intermediate Sensitive	Sensitive
Penicillin	≤ 26	27-46	≥47
Tetracycline	≤30	31-37	≥38
Spectinomycin	≤14	15-17	≥18
Ceftriaxone	-	-	≥35
Cefixime	-	-	≥31
Ciprofloxacin	≤27	28-40	≥41
Azithromycin	≤30	-	-

1.8.1.2 Calibrated Dichotomous Sensitivity (CDS) test.

Materials required:

- Inoculating wire: a straight nichrome wire (Nichrome SWG24, 0.56 mm diameter). The wire should be 10 cm in length and cut with sharp scissors so that end is square.
- Loop holder for wire.
- 2.5 ml sterile normal saline in test tubes.
- Rubber teats, Pasteur pipettes.
- Clear plastic ruler (mm) or vernier calipers.
- Incubator set at a temperature of 35°C - 37°C.
- CO₂ source.- Jar or incubator

Medium:

Chocolate agar comprising Columbia agar base are suitable. 20 ml agar is dispensed into a 90 mm Petri dish so that depth of agar in each plate is approximately 4 mm.

The plates are stored in an inverted position in the refrigerator before use and are used within 7 days of pouring.

On the day of test, the plates are dried by inverting them without the lid in an incubator at 35°C - 36.5°C for 45 minutes to an hour.

Inoculum:

1. The inoculum is prepared from a typical colony of at least 2 mm diameter grown overnight on chocolate agar.
2. After flaming and cooling the wire, the inoculum (10^7 cfu/ml) is prepared as follows:
 - ▶ Pass the straight wire through a colony until it touches the surface of the agar. Move the wire across so that most of the colony is picked up. Bacterial material must be visible on the tip of the straight wire after removal.
 - ▶ Where possible, the inoculum should be obtained by stabbing to get a single isolated colony. However, if only small colonies are available it may be necessary to collect 3-5 colonies before the material is visible.
 - ▶ Inoculate in a test tube containing 2.5 ml saline by rotating the straight wire at least 10 times with the tip touching the bottom of the test tube.
 - ▶ Make sure that the material has come off from the tip into the saline. Some gonococci produce sticky colonies and the material may have to be teased apart.

Inoculation of plates:

1. Mix the inoculum using a Pasteur pipette at least 10 times and check there are no lumps left in the suspension.
2. Flood the dried chocolate agar plate and remove the excess inoculum.
3. Allow the plate to dry at room temperature. This may take 10-15 minutes. Plates must not be left longer than 15 minutes after the inoculum has dried.
4. Apply antibiotic discs using sterile forceps. Ensure that discs are applied evenly but do not reapply discs after initial contact with the agar.
5. Low concentration discs are recommended in this method. Upto 6 discs can be applied on a single 90 mm diameter plate.
6. Incubate the plates at 35°C - 36°C in air containing 5% CO_2 and 70 to 80% humidity for 18 hours.

Procedure:

1. Sample colony with a straight wire
2. Prepare a suspension in 2.5 ml normal saline.
3. Inoculate a pre-dried chocolate agar plate.
4. Distribute the inoculum by rocking.
5. Remove excess inoculum.
6. Dry the plate at room temperature.
7. Load the plate with antibiotic discs.
8. Incubate for 18 hours.
9. Measure the annular radii.
10. Interpret zone sizes.

Reading zones (annular radius):

The annular radius (mm) is the shortest distance from the edge of the disc to the edge of confluent growth (Because chocolate agar plates are used, zone sizes must be measured from the inoculated surface)

Disc strengths, Annular radius Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) for *Neisseria gonorrhoeae*.

Antimicrobial Agent	Disc Content	Annular Radius (mm)			Equivalent MIC (mg/l)		
		Resistant	Less sens	Sensitive	Resistant	Less sens	Sensitive
Penicillin¹	0.5 IU	< 3mm	3 – 9mm	> 9mm	≥ 1.0	0.06 - 0.5	≤ 0.03
Quinolone testing²							
Ciprofloxacin	1 µg	≤ 6mm	> 6mm	>6mm	≥ 1.0	0.06 - 0.5	≤ 0.03
Naladixic acid	30 µg	0mm	0mm	>6mm			
Ceftriaxone	0.5 µg	–	5-9 mm ³	> 9mm	–	0.03- 0.25	<0.03
Cefpodoxime	10 µg	–	≤ 12	> 12			
Spectinomycin	100µg	0mm	–	≥ 6mm	≥ 128		≤ 64
Tetracycline	10 µg	TRNG ≤ 1 mm	or	Not TRNG ≥ 2 mm	TRNG ≥ 16 CMRNG 2-8	0.5-1	≤ 0.25
Azithromycin	15 µg	< 8 mm		≥ 8mm	≥ 1.0		≤ 0.5

^{1.} The detection of beta-lactamase production defines a gonococcus as a PPNG and as resistant to the penicillins. Penicillin disc testing of PPNG is optional, usually no zone of inhibition is present.

^{2.} Quinolone disc testing is performed using a combination of both ciprofloxacin 1 µg (a fluoroquinolone) and nalidixic acid 30 µg (a quinolone) discs. Susceptibility to nalidixic acid is not reported but is used in determining susceptibility to ciprofloxacin. Categories of susceptibility to ciprofloxacin are defined as follows:

▶ **Susceptible to ciprofloxacin.**

The annular radius of the inhibitory zone around both nalidixic acid 30 µg and ciprofloxacin 1 µg discs is >6 mm.

▶ **Less susceptible to ciprofloxacin**

The annular radius of the inhibitory zone around a nalidixic acid 30 µg disc is < 6 mm (usually

0 mm) and that around a ciprofloxacin 1 µg disc is > 6 mm. These isolates are considered less susceptible to ciprofloxacin with an MIC of between 0.06 mg/L and 0.5 mg/L.

▶ **Resistant to ciprofloxacin**

The annular radius of the inhibitory zone sizes around a ciprofloxacin 1 µg disc is ≤ 6 mm.

The annular radius around a nalidixic acid 30 µg disc will also be < 6 mm (usually 0 mm).

The result obtained for ciprofloxacin susceptibility can be extrapolated to other fluoroquinolones.

Doubtful results can be repeated with use of controls or checked by MIC method.

Reference: Antibiotic susceptibility testing by CDS Method. S.M.Bell, J.N.Pham, TT Nguyen, 2012 Sixth Edition. <http://web.med.unsw.edu.au/cdstest.html>

1.8.2 MIC testing can be performed by E test or agar dilution method depending on facilities available in the laboratory.

1.9 Quality control for antibiotic sensitivity testing

Media:

Check the approximate depth of agar and weight of agar plates. Test the control strains and check that the zone sizes are within the acceptable range and make sure that a good lawn growth is obtained.

Inoculum:

Bacterial material must be seen on the tip of the straight wire. After overnight incubation there must be confluent growth on the sensitivity plate.

Potency of antibiotic discs:

Check that the correct disc potency is used. The following precautions should be taken while handling the discs:

- ▶ Long term storage: at -20°C in tightly sealed, desiccated container.
- ▶ Short term storage (≤ 1 week): 2-8°C in tightly sealed desiccated container.
- ▶ Discs must be allowed to warm to room temperature before opening the container to avoid condensation on the discs as this will inactivate some antibiotics, e.g. benzyl penicillin.
- ▶ Do not use discs past their date of expiry

Incubation temperature and atmosphere:

The temperature should be monitored using a maximum/minimum thermometer which should be checked daily, especially after incubation of the plates. Air should also be monitored for humidity and concentration of CO₂ (5%-7%).

Reference strains

Set up antimicrobial susceptibility testing at weekly intervals, with WHO standard /ATCC strains.

Initially, reference strains should be tested for 20 consecutive days to ensure that laboratory procedure followed is correct and reproducible consistently. Subsequently weekly quality controls should be run. In addition, whenever there is a change in the lot no of disks, quality checks should be done. If there is a change in the manufacturer of disks, QC should be done for ten consecutive days before being put to regular use.

Clinical isolates should be sent to WHO Regional Reference Laboratory for Gonococcal Antimicrobial Surveillance Programme (GASP) at Vardhman Mahavir Medical College and Safdarjung Hospital for confirmation of antimicrobial susceptibility testing results.

REFERENCE STRAINS

Table. MIC and category of sensitivity of the 2008 WHO *Neisseria gonorrhoeae* reference strains panel, intended for global quality assurance and quality control of antimicrobial resistance testing and surveillance.

Characteristics	WHO F	WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P
β-lactamase production	-	-	-	-	Pos	Pos	Pos	-
Penicillin G (0.032->32) ^a	S (0.032)	I (0.5)	CMR ^b (2)	CMR ^b (2)	PPNG ^b (8)	PPNG ^b (8)	PPNG ^b (>32)	I (0.25)
Ampicillin (0.032-24) ^a	S (0.032)	I (0.25)	I (2)	I (2)	PPNG ^b (8)	PPNG ^b (4)	PPNG ^b (24)	S (0.064)
Cefuroxime (0.064-12) ^a	S (0.064)	I (0.5)	R (12)	R (8)	I (0.5)	I (0.25)	I (1)	I (0.125)
Cefixime (<0.016-0.5) ^a	S (<0.016)	S (<0.016)	NS ^c (0.5)	NS ^c (0.25)	S (<0.016)	S (<0.016)	S (0.016)	S (<0.016)
Ceftriaxone (<0.002-0.25) ^a	S (<0.002)	S (0.008)	NS ^c (0.064)	NS ^c (0.125)	S (0.012)	S (0.004)	S (0.032)	S (0.004)
Ertapenem ^d (0.004-0.125) ^a	S (0.004)	S (0.008)	NS ^c (0.125)	NS ^c (0.064)	S (0.012)	S (0.008)	S (0.032)	S (0.008)
Erythromycin (0.5-4) ^a	S (0.5)	I (1)	I (1)	I (2)	I (1)	S (0.5)	I (1)	R (4)
Azithromycin (0.125-2) ^a	S (0.125)	S (0.25)	S (0.25)	I (0.5)	S (0.25)	S (0.125)	S (0.25)	R (2)

Characteristics	WHO F	WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P
Ciprofloxacin (0.004->32) ^a	S (0.004)	LLR ^e (0.125)	HLR ^e (>32)	HLR ^e (>32)	R (2)	R (4)	S (0.008)	S (0.004)
Spectinomycin (16->1024) ^a	S (32)	S (16)	S (16)	S (16)	S (16)	S (16)	R (>1024)	S (16)
Kanamycin ^d (8-32) ^a	S (16)	S (16)	S (16)	S (32)	S (16)	S (16)	S (16)	S (8)
Gentamicin ^d (2-8) ^a	S (4)	S (4)	S (2)	S (8)	S (4)	S (4)	S (4)	S (4)
Tetracycline (0.25-32) ^a	I (0.25)	TRNG ^f (32)	R (2)	R (4)	I (1)	TRNG ^f (16)	I (1)	I (0.5)
Rifampicin ^d (0.125->32) ^a	S (0.125)	S (0.5)	S (0.5)	S (0.5)	R (>32)	R (>32)	S (0.25)	R (>32)

^a category of sensitivity based on minimum inhibitory concentration (MIC, mg/l) using Etest. The range of the MICs for each antimicrobial and the different strains are given in parenthesis. Most important, the precise MICs should be interpreted with caution because these were derived using one specific method only and, accordingly, can slightly differ using other methods. However, the identified resistance phenotypes (SIR-categorization) should be the same.

^b CMR, chromosomally-mediated resistance; PPNG, penicillinase-producing *N.gonorrhoeae* that should always, independent of identified MIC-value, be considered resistant to penicillins.

^c NS, non susceptible, contains genetic resistance markers, but clinical/laboratory correlates are insufficient to allow resistance phenotype designation.

^d Breakpoints from other sources than the Swedish Reference Group on Antibiotics (www.srga.com): ertapenem, tentative based on wild type MIC-distribution; kanamycin; gentamicin; and rifampicin (www.bsac.org.uk).

^e LLR, low-level resistance; HLR, high-level resistance.

^f TRNG, tetracycline resistant *N.gonorrhoeae* (plasmid-mediated)

Reference: Unemo, M, Fasth O, Rfdlund H, Limnios A, Tapsall J, Phenotypic and genetic characterization of 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purpose. J Antimicrob Chemother, 2009; 63;1142-51

Expected values for quality control strain ATCC 49226 are:

Antimicrobial agent	Disk content	Expected range in mms
Penicillin	10IU	26-34
Ceftriaxone	30µg	39-51
Cefepime	30µg	37-46
Ciprofloxacin	5µg	48-58
Tetracycline	30µg	30-45
Spectinomycin	100µg	23-29

Table. Category of sensitivity of the reference strains of *N. gonorrhoeae* for quality control in AGSP by MIC and CDS technique.
(courtesy Athena Limnios WHO-CC Sydney)

DISC TYPE and POTENCY REFERENCE STRAINS	Penicillin			Ciprofloxacin			Ceftriaxone			Spectinomycin			
	MIC mg/1	P 0.5u Disc An. Radius Mm	Category	MIC mg/1	Cip 1µg Disc An. radius mm	N.A. 30µg Disc An. radius mm	Category	MIC mg/1	Cro 0.5µg Disc An. radius mm	Category	MIC mg/1	SH 100µg Disc An. radius mm	Category
1. WHO C	0.5	4mm	Less sensitive	0.016	16mm	13mm	Sensitive	0.016	10mm	Sensitive	<, ¹ =64	8mm	Sensitive
2. WHO G	0.5	4mm	Less sensitive	0.25	9mm	0mm	Less Sensitive	0.008	11mm	Sensitive	<, ¹ =64	9mm	Sensitive
TRNG ⁴													
3. WHO L	2	0mm	Resistant (CMRNG) ¹	16	0mm	0mm	Resistant HLR ³	0.125	6mm	Less sensitive	<, ¹ =64	8mm	Sensitive
4. WHO O	32	0mm	Resistant (PPNG) ²	0.03	15mm	13mm	Sensitive	0.016	12mm	Sensitive	>1024	0mm	Resistant
5. WHO Q	1	0mm	Resistant (CMRNG) ¹	1	2mm	0mm	Resistant	<, ¹ =0.03	8mm	Sensitive	<, ¹ =64	8mm	Sensitive
TRNG ⁴													

1. CMRNG= Chromosomally mediated resistant Neisseria gonorrhoeae
2. PPNG = Penicillinase producing Neisseria gonorrhoeae
3. HLR = High Level Resistance
4. TRNG = Plasmid mediated resistance to Tetracycline (WHO G and WHO Q are TRNG strains)

Table. Australian Gonococcal Surveillance Programme Quality Control values for antimicrobial susceptibilities of reference strains of *Neisseria gonorrhoeae* determined by MIC, agar plate dilution test method.

Antimicrobial Agen	Disc Content	Minimal Inhibitory Concentration (MIC mg/l) and Category of Sensitivity													
		N. gonorrhoeae Reference Strain →	WHO A	WHO C	WHO E	WHO G	WHO J	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	WHO Q	
		Phenotypic Category ¹ →	PenSENS SpecRES	PenLS	PPNG	PenLS LSCRNG TRNG	PPNG RQRNG	PenRES RQRNG CeftriLS	PenRES RQRNG CeftriLS	PPNG RQRNG	PPNG RQRNG TRNG	PPNG SpecRES	PenLS AzithRES	Pen R RQRNG	
Penicillin	0.5 unit	MIC ² Category ³	0.008 S	0.5 LS	>2.0 PPNG	0.5 LS	>2.0 PPNG	2.0 R	2.0 R	>2.0 PPNG	>2.0 PPNG	>2.0 PPNG	0.25 LS	1.0 R	
Ciprofloxacin	1 µg	MIC Category	0.016 S	0.016 S	0.016 S	0.25 LS	8 HLR	>16 HLR	16 HLR	2 R	4 HLR	0.03 S	0.016 S	1.0 R	
Ceftriaxone	0.5 µg	MIC Category	<, ¹ =0.008 S	0.016 S	<, ¹ =0.008 S	<, ¹ =0.008 S	<, ¹ =0.008 S	0.06 LS	0.125 LS	<, ¹ =0.008 S	<, ¹ =0.008 S	<, ¹ =0.008 S	<, ¹ =0.008 S	<, ¹ =0.03 S	
Spectinomycin	100µg	MIC Category	512 R	<, ¹ =64 S	<, ¹ =64 S	<, ¹ =64 S	<, ¹ =64 S	<, ¹ =64 S	<, ¹ =64 S	<, ¹ =64 S	<, ¹ =64 S	>1024 R	<, ¹ =64 S	<, ¹ =64 S	
Tetracycline	10 µg	MIC Category	<, ¹ =8 Not TRNG	<, ¹ =8 Not TRNG	<, ¹ =8 Not TRNG	32 TRNG	<, ¹ =8 Not TRNG	<, ¹ =8 Not TRNG	<, ¹ =8 Not TRNG	<, ¹ =8 Not TRNG	16 TRNG	<, ¹ =8 Not TRNG	<, ¹ =8 Not TRNG	>32 TRNG	
Azithromycin	-	MIC Category	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	<, ¹ =0.5 S	2 R	<, ¹ =0.5 S	
B-lactamase			Negative	Negative	Positive	Negative	Positive	Negative	Negative	Positive	Positive	Positive	Negative	Negative	

S = Sensitive; LS = Less sensitive; R = Resistant; PPNG = Penicillinase producing *N. gonorrhoeae*; TRNG = Tetracycline resistant *N. gonorrhoeae* (High level tetracycline resistance, plasmid mediated).

Reference: Tapsall J and Members of the National Neisseria Network of Australia. Antimicrobial testing and applications in the pathogenic *Neisseria*. In: merlino J, ed., Antimicrobial susceptibility testing: methods and practices with an Australian perspective. Australian Society for Microbiology, Sydney, 2004. Pp 175-188.

1.10 Maintenance of bacterial strains

Reference strains of designated categories of susceptibility should generally be maintained by the laboratory. The laboratory may also store certain clinical isolates of interest for reference. The following storage methods may be used in the absence of facilities for storage by liquid nitrogen.

1.10.1 Preservation of bacteria by deep freezing (-70°C):

Any of the following two methods can be used for preservation of *Neisseria gonorrhoea* at -70°C for a long time.

a. Nutrient broth plus 20% glycerol

Glycerol 20 ml
Nutrient broth 80 ml

Sterilize by autoclaving at 15 psi (121°C) for 15 minutes. The medium used is distributed in 0.7-1.0 ml aliquots in small test tubes and stored at 2-8°C prior to use.

Preparation of bacterial suspensions

A heavy inoculum of bacteria is made in 0.7-1.0 ml nutrient broth with glycerol. Using the sample pipette, the suspension is mixed well and then transferred to a labeled cryo vial before placing in a freezing box at -70°C.

b. Preservation Medium

Ingredients

1. Trypticase Soy Broth, Becton Dickinson 30 g
2. Yeast Extract 3.0 g
3. Agar No 2, Lab M 0.5 g
4. Distilled water 700 ml
5. Horse serum 300 ml

Method

1. Mix 1-4
2. Sterilise at 121°C for 15 mins
3. Cool Mixture to <50°C
4. Add horse serum and mix
5. Check pH and if needed, adjust pH to 7.5 ± 0.1
6. Dispense in sterile tubes for freezing, volume 1ml
7. Store at 2-8°C

Preparation of bacterial suspension

Subculture on chocolate agar and make suspension from fresh 20-24 hr subculture. Use all the material from a 90mm agar plate into 0.5-1ml of above medium.

Recovery of cultures

Remove the cryo tube from the freezer but do not allow it to thaw. Using the tip of a Pasteur pipette, aseptically remove a small sample of the frozen suspension and inoculate a suitable medium. Replace the cryotube in the freezer.

Note: Record how often a tube is accessed for subculture.

1.10.2 Preservation on chocolate agar slopes:

Strains of *N.gonorrhoeae* may be stored on chocolate agar slopes overlaid with paraffin oil for upto 3 months or more until a more permanent means of storage is undertaken. (Reference Method: WHO CC Sydney)

Procedure: A fresh, pure culture of the gonococcus to be stored is heavily inoculated onto a 3 ml volume chocolate agar slope in a polycarbonate (plastic) screw-top Bijou (5 ml volume) bottle and incubated with the screw cap loosened for a minimum of 24 hours in a CO₂ enriched atmosphere or until visible growth is present on the agar surface. Sterile liquid paraffin is then used to completely fill the agar slope, the screw cap lid is fully tightened and the Bijou bottle slope is then stored at 35-36°C until the *N.gonorrhoeae* are tested or forwarded elsewhere. When the gonococci are required for testing, a sterile bacteriological loop is inserted through the paraffin overlay to remove the bacterial growth. When this is inoculated onto a fresh agar plate and streaked, globules of paraffin will also be present. However after incubation, for 48 hours, gonococcal colonies are readily discernable and can be subcultured for appropriate examination. The original paraffin overlaid slope can be returned to storage for further use, but special care must be taken at the initial inoculation to use a pure culture and to ensure that when sampled the slope is not contaminated.

Documenting location of strains: This should be recorded as should details of access to the slopes.

Note:

- i) polycarbonate (plastic) bottles, not glass, must be used.
- ii) larger volume containers may also be used e.g. 30 ml MacCartney bottles but these should also be polycarbonate and will require larger volumes of bacteriological media and paraffin and occupy more storage space.
- iii) the storage temperature should be 36°C, not room temperature, or else loss of viability will occur,
- iv) for transport, lower temperatures will not affect viability for transit times of up to 5 days
- v) loss of plasmids will occur on long term storage;
- vi) it is suggested that familiarity with the system be obtained by both recipients and receivers of the slopes before the system is implemented.
- vii) fully viable gonococci have been recovered after nine months with this system, but there are no data on viability beyond this period.
- viii) paraffin may be sterilized in large batches e.g. 200 ml placed in a hot air oven at 180°C for 2 hours.
- ix) it is important that the slopes be completely filled with paraffin and that there are no air bubbles.

- x) viability decreases substantially if the slopes are not stored at 36°C.
- xi) careful techniques must be maintained with respect to sterility at all times when subculturing.

1.10.3 Lyophilization:

Prepare 7.5% glucose horse serum (7.5 gm of glucose to 100 ml horse serum) sterilize and put 1 ml in each test tube. Inoculate each tube with a very heavy growth from half a plate, vortex, distribute into 3 ampoules, freeze and lyophilize.

Method for Resuscitating Freeze-dried cultures of *Neisseria gonorrhoeae*

1. Vials containing lyophilised *N. gonorrhoeae* should be protected from light and stored in the refrigerator (4°C) until required.
2. The reconstituting media used is 1 ml of a nutrient blood broth or a rich peptone broth supplemented with 10% blood. (eg. 1ml nutrient broth plus 5 drops of blood). Allow broth to come to room temperature before use/alternatively use BHI broth and keep the suspension for 1 hour in CO₂ incubator / candle jar at 37°C.
3. Working in a safety cabinet, open vial by applying gentle pressure at the neck of vial.
4. Add 1 ml of the blood broth to the vial and carefully reconstitute all visible material from the wall of the vial and allow the broth to soak into the beads.
5. Place two drops of suspension onto culture plate for the growth of *N. gonorrhoeae*, streak out and incubate.
6. Transfer the remaining broth and beads into a test tube and mix again.
7. Incubate the test tube as this may be subcultured the following day if the direct culture plate fails to show growth.

1.11 Nucleic acid amplification test

Many laboratories with facilities for molecular testing employ NAATs with very high reliability. Swabs are to be transported from periphery as per user's protocol and tested in reference laboratories. Multiplex PCR for simultaneous detection of *N.gonorrhoeae* and *C.trachomatis* has been widely in use and is well validated for routine use.

SUMMARY

1. Direct microscopic examination of Gram stained slides is done at the peripheral level. More than 95% of the males with symptoms can be diagnosed by grams staining alone. Grams stain is not a recommended method of diagnosis in female patients.
2. Specimens from women and men should be cultured, preferably on two media, one with inhibitors and one without it. For all cultures, colony morphology, oxidase reaction, superoxol test and Gram negative diplococci in smear made from culture and rapid carbohydrate utilization test (RCUT) confirm the diagnosis. β-Lactamase test can also be done.
3. Antibiotic sensitivity testing should be done at central level regularly.

2. CHLAMYDIAL INFECTIONS

Chlamydia trachomatis is an intracellular organism. It causes lower genital tract infection, such as non-gonococcal urethritis and cervicitis, and may cause PID and endometritis (serovar D to K). It can cause inclusion conjunctivitis. Three specific serovars (L1 to L3) cause lymphogranuloma venereum (LGV), while other serovars (A to C) cause trachoma. Smear and staining do not help in the diagnosis. Tissue culture (gold standard), antigen detection (DFA and ELISA), nucleic acid hybridization and amplification methods are used for laboratory diagnosis.

The current reference test (expanded gold standard) for diagnosing *C.trachomatis* infection is commonly consistent result with two non-culture techniques. The culture procedure, however, is expensive, slow, labour-intensive, technically difficult and beyond the capacity of most laboratories. Culture is not suitable for widespread screening and is mostly carried out in research laboratories. In competent hands, the specificity of culture is 100 percent, its sensitivity is estimated to be no more than 70 to 85 percent compared to DNA amplification. Cervical culture for *C.trachomatis* has a 65-90 percent sensitivity compared to DNA amplification of first-catch urine. Other non-culture techniques (DFA, EIA, DNA hybridisation) developed during the 1980s, which are easier to perform and less expensive than culture, are still widely used.

2.1 Available diagnostic tests and samples to be collected for *C.trachomatis*

Test	Method	Sample
DFA (Direct Fluorescent antibody)	Microscopy	Urethral, endocervical, rectal swabs
EIA (Antigen detection)	Immunochromatography	Urine, urethral, endocervical swabs
Culture	Tissue culture	Cervical swabs, pus from bubo
DNA probe	NAH (Nucleic Acid Hybridization)	Urethral, endocervical swabs
PCR	NAAT	Urine, urethral, endocervical swabs

2.2 Transport and processing of specimens:

Cervical and Urethral Swabs - shake the swab in the phosphate buffered sucrose transport medium (Annexure-II), press the swab to the wall of the bottle to remove liquid from it and then discard the swab.

Bubo pus - viscous material should be ground and suspended in nutrient broth or cell culture medium. This should be tested for bacterial contamination and then inoculated into cell cultures for isolation of Chlamydia.

Rectal swabs - As they are likely to be contaminated with gut flora, suspend the swabs in cell culture medium containing gentamycin, vancomycin. Shake the suspension to mix, centrifuge for 10 mins at 300g. Inoculate supernatant onto cell culture medium.

Tissue samples for culture - to be transported in dry ice in a frozen condition to central laboratory. Thaw, mince with sterile scissors, grind with a homogeniser. Add cell culture medium to make a 20% suspension and incubate.

2.3 Test Methods

1. Isolation in cell culture

Culture is the only procedure that confirms the presence of viable organisms. Antigens, nucleic acids or antibodies can be present in the absence of viable infectious particles.

Transport swabs at 4°C in ice within 4 hours of collection. If it cannot be cultured within 48 hours, freeze at -70°C.

Mc Coy, Hep2 or Hela cells are commonly used for culture of Chlamydia. Before inoculating samples, pretreat the cell monolayers with 30µg/ml of Diethylaminiethyl-Dextran in Hank's balance salt solution for 20 mins. Clinical specimens are shaken with sterile 5 mm glass beads to lyse the epithelial cells and release the chlamydiae before being used for inoculation. For inoculation, the medium is removed from the cell monolayer and 0.1 mL to 1 mL of inoculum is added to the cells. The specimen is centrifuged onto the cell monolayer at approximately 3000 g at room temperature for 1 h.

Shell vials are incubated at 35°C in 5% CO₂ for 2 h to allow for the uptake of chlamydiae. The medium is then discarded and replaced with medium containing 1 µg of cycloheximide/mL. The cells are incubated at 35°C in 5% CO₂ for 48 h to 72 h, and one cover slip is examined for inclusions by immunofluorescence, iodine staining or Giemsa staining. Immunofluorescence is the preferred method because it is more specific than Giemsa staining and can give a positive result as early as 24 h post inoculation.

2. Non-culture diagnostic techniques

2.1 Direct Microscopy

Gram staining:

Gram stain of a urethral specimen from men with urethral discharge demonstrating polymorpholeukocytes without the presence of Gram-negative diplococci is strongly indicative of chlamydial infection.

Giemsa staining:

Giemsa staining has very poor sensitivity.

2.2 Antigen detection - Direct Fluorescent Antibody (DFA) technique:

- 1) Commercial kits are available to do the test. Only a broad outline of the technique is described here.
- 2) Roll the sample swab in the well on the Teflon coated glass slide.
- 3) Air dry the smear and fix with acetone or methanol.
- 4) Put 30µl of fluorescein labeled *C.trachomatis* species specific monoclonal antibody on the smear.
- 5) Incubate the slide in moist chamber at room temperature for 15 min.
- 6) Wash the slide with distilled water for 20 sec.
- 7) Air dry the slide.
- 8) Put 50% PBS– 50% glycerol mounting solution on the well and place a cover slip on the well.
- 9) See under fluorescent microscope at 500 x magnification.

Reading:

Chlamydial elementary bodies - Bright apple-green pinpoints.
Cells - Red

Interpretation:

Positive: > 10 Elementary Bodies / slide
Doubtful positive: < 10 Elementary Bodies / slide
Negative: No Elementary Bodies

Actual instructions for the test procedure are supplied with the kit to be used and should be followed as such. This is the only test available for simultaneous assessment of specimen adequacy by visualization of cuboidal columnar epithelial cells and is sensitive and specific in symptomatic patients.

2.3 Antigen detection by Enzyme Immuno Assays

A number of commercial EIAs are available for the detection of chlamydial antigens in clinical specimens. Details of each step are given in the kit to be used. Therefore, follow the

manufacturer's instructions. These methods have a lower sensitivity than the DFA test.

3. Non-nucleic acid amplification tests (Non-NAATs) or Nucleic acid probe hybridization tests (NAH)

NAH tests detect specific DNA or RNA sequences of *C.trachomatis*. These tests require urethral swab specimens and are 10% to 15% less sensitive than NAATs.

4. Nucleic acid amplification tests (NAATs):

NAATs are recommended by CDC for diagnosis of genital and extragenital infections and for screening. These can be used for less invasive samples, are highly sensitive with self-obtained samples (e.g. vaginal swabs or urine) and are being used for extragenital samples. Results obtained by DNA amplification techniques on urine specimens from both men and women have shown that this approach is at least as good as cell culture on classic genital swab specimens. However, the high cost of these assays and the requirement of specialised laboratory equipment currently limit the use of these methods in routine diagnosis. Roche Amplicor, LCX by Abbott, APTIMA, ProbeTec are some of the NAATs that are available for amplification and detection of chlamydial nucleic acid. Procedures as per the manufacturer's instructions should be followed.

Internationally approved NAATs are recommended for use and in-house tests should be validated against internationally approved NAATs. The facilities for these should be established in regional reference laboratories for diagnostic purposes because of their higher sensitivity and specificity. Different type of genes such as MOMP gene, Plasmid DNA, r-RNA - 16S & 23S and phospholipase gene are targeted for diagnosis by NAATs. Some of the NAATs targeting the common endogenous plasmid can give false negative results with a new variant of *C.trachomatis* (nvCT) strain which has been recently isolated in Sweden (2006) and this variant has a 277 bps deletion in a portion of the plasmid. Therefore, the choice of sequence is crucial.

Multiplex PCRs to detect *C.trachomatis* and *N.gonorrhoeae* are useful for detection of both these organisms with good sensitivity and specificity.

5. Point of care (POC) tests:

POC tests are newer easy-to-use tests with EIA technology in formats based primarily on membrane capture or latex immunodiffusion. These enable rapid diagnosis of chlamydial infection under field conditions. However, their sensitivity is low (50-60%) but are highly specific (>90%) and not to be used in low-prevalence population

SUMMARY

1. Smear examination is not helpful for diagnosis of genital Chlamydia infection and hence should not be done.
2. Chlamydia culture is the best method for diagnosis (gold standard), but should be done at the central references laboratories. PCR has now replaced the culture.
3. Antigen detection method is good in central/intermediate laboratories with trained staff and fluorescent microscope, or ELISA reading equipment.
4. Antibody detection has only limited use.
5. The term expanded gold standard/ defined reference standard, i.e., commonly consistent result with two non-culture techniques is considered to be a useful tool for diagnostic purposes.

3. SYPHILIS

Syphilis is caused by the bacterium, *Treponema pallidum*, which is a Spirochaete. It occurs due to the contact of an infectious lesion with the mucous membrane or injured skin. Culture of *T.pallidum* is not feasible. So the clinical diagnosis of syphilis is confirmed by i) seeing the organism in the exudate from ulcers; or ii) by finding antibodies to this pathogen in serum or cerebrospinal fluid.

Classical syphilis occurs in stages- Primary, Secondary and Tertiary stages, with a latent stage in some cases. In the early stage of the disease (primary syphilis) *T. pallidum* can be seen in ulcers in the genital or rectal area. Antibody can be detected from 1 to 4 weeks after the formation of the ulcer, therefore, seeing the organism in the lesion provides the most reliable method of diagnosis in the early stage of the infection.

3.1. Test sample requirements

Test	Method	Sample
Demonstration of organism	Microscopy-Dark Field, Direct Fluorescence	Serous fluid from ulcers, tissues
Non-treponemal tests	RPR, VDRL	Blood (Serum)
Treponemal tests	Immunochromatography, TPHA, FTA-Abs	Blood (Serum)
NAAT-PCR	Molecular	Tissue, serous fluid

3.2. Dark Field Microscopy for detection of *Treponema pallidum*

Test Principles

Clinical diagnosis of primary syphilis is confirmed by using dark field microscopy to demonstrate *Treponema pallidum* in material from suspected lesions. A positive result is an almost certain diagnosis of syphilis.

Specimen Collection

Materials required:

1. Dark field microscope
2. Thin glass slides (1mm)
3. Thin cover slips (.1mm)
4. Glass marking pencil / marker
5. Normal Saline

The ideal specimen for dark field examination is a serous fluid that is rich in *T.pallidum* but that contains few blood cells (treponemes may be obscured if many cells are present). Consider every genital lesion in sexually active patients as syphilis until subjected to a dark field examination and proven otherwise.

Dark field examination of oral lesions is not recommended. All positive dark field tests with mouth specimens must be confirmed by a direct fluorescent antibody test. The indigenous flora of the oral cavity frequently contain a spiral organism, *T. denticola*, which is indistinguishable from *T. pallidum*. It can give false-positive results.

1. Collection from genital ulcers

- a. Remove any scab or crust covering the lesion.
- b. Secondary infection exudate, if any, should be removed with a gauze sponge.
- c. If necessary, compress the base of the lesion or apply a suction cup to the lesion to promote the accumulation of tissue fluid on the ulcer surface.
- d. Apply a glass slide to the oozing lesion or use a sterile bacteriological loop to transfer the fluid from the lesion to the glass slide.
- e. Place a cover glass making sure to remove air bubbles
- f. Examine the slide immediately (within 5-20mins) Any appreciable delay in examining a specimen may result in questionable findings, because the motility of the treponemes may be reduced or completely lost.
- g. To prevent drying, place additional slides with specimens in a moist chamber such as a large plastic petri dish containing a moistened paper towel.

Note: The slide preparations should not contain a large volume of fluid (large volumes cause a rapid liquid flow across the field), nor should the preparation be so thin that it begins to dry before an adequate examination can be made.

2. Dry papulo squamous lesions of the skin

- a. Gently remove the superficial layer of skin with a scalpel, needle tip, or mechanical abrader.
- b. Try not to cause bleeding. If very little serous fluid appears, compress the lesion.
- c. Touch the corner of the surface of a microscope slide to the fluid or use a sterile bacteriological loop to transfer the material to the slide.
- d. Material can also be collected by injecting a small drop of sterile saline into the base of the lesion and aspirating the fluid with a thin needle and syringe.

3. Cervical/vaginal lesions

- a. With visualization by a bivalve speculum, remove any cervical or vaginal discharge.
- b. Obtain serous exudate with a sterile bacteriological loop.
- c. Prepare slides as described above.

4. Mucous patches

- a. Using a sterile bacteriological loop, collect some of the mucous material and place it on a clean glass slide.
- b. Place a cover glass on the specimen and examine immediately.

5. Lymph node

- a. Disinfect the skin over the node by swabbing it with iodine and alcohol or another suitable agent.
- b. Rinse a sterile 20 gauge needle and a 2ml syringe with sterile physiological saline.
- c. Allow 0.2 ml or less of the saline to remain in the syringe.
- d. Hold the node firmly and insert the needle well into the node. The ability to manipulate the node freely with the needle tip is a good indication that the capsule of the node has been pierced.
- e. Inject the sterile physiological saline into the node.
- f. Macerate the tissue by gently manipulating the needle in various directions.
- g. Aspirate as much fluid as possible.
- h. Discharge the aspirated material onto slides for immediate examination.

Adjustment of the Microscope for Dark field Examinations

Microscope should be adjusted and in satisfactory working condition BEFORE collecting the specimen for examination.

Prepare a control slide by placing 1 drop of a suspension of *T. phagedenis* or a drop of saline plus gingival scrapings on a microscope slide of appropriate thickness.

1. Press the slide on the fluid.
2. Put a drop of normal saline on the fluid on the slide and mix well.
3. Collect the fluid at the edge of a cover slip, and place the cover slip on the material.
4. Seal the edges of the cover slip with petroleum jelly/nail varnish.
5. Bring the condenser of the darkfield microscope down slightly.
6. Put a drop of immersion oil on the condenser of darkfield microscope.
7. Keep the slide on microscope stage.
8. Bring the condenser up so that oil touches the undersurface of the slide.
9. Examine the slide immediately.
10. Make sure that there is no air bubble in the oil.
11. Focus the specimen under low power objective (10x).
12. Focus the condenser to get a very small diameter of light in the centre.
13. Next focus the slide under 40 x magnification (dry).
14. Carry out the microscopy in a dark room to get good results.
15. The slide may also be observed under oil immersion (100x) after adding a drop of oil on the coverslip.

Reading:

- ▶ In a black background *T.pallidum* is seen as a silvery-white and bright, a thin, spiral organism, and 6-14um long, with 8-14 spirals which are pronounced and regular.
- ▶ It moves by rotation, bending and twisting and increasing and decreasing in length.
- ▶ **Presence of *T. pallidum* confirms the clinical diagnosis of syphilis.**

After examining a slide, discard it into a container of 70% alcohol or other suitable bactericidal solution such as 1% sodium hypochlorite.

3.3. Direct fluorescent antibody test for *T. pallidum*

The direct fluorescent antibody test for *T.pallidum* is easier to perform than dark-field microscopy. Since antigen is detected, it does not require the presence of motile treponemes.

This test uses fluorescein isothiocyanate-labelled antibody specific to pathogenic treponemes, and therefore is suitable for the examination of specimens from oral and rectal lesions.

However, this test does not differentiate between *T. pallidum* and other pathogenic treponemes causing yaws, endemic syphilis and pinta.

For detailed steps, the laboratory is advised to follow manufacturer's instructions. As the test requires fluorescent microscope, this test would not be available at primary level. This may be done at intermediate level laboratories.

3.4. Serological tests:

These demonstrate antibodies to *T.pallidum*. Two types of serological tests are available for the diagnosis of late primary, secondary and late syphilis. The non-treponemal or standard tests for syphilis (STS) detect the presence of non-specific anti-cardiolipin antibodies (Reaginic antibodies) in the serum. The other group of tests called the specific, or treponemal tests, detect the antibodies specific for pathogenic treponemes in the serum.

For collection of blood, please refer to chapter on sample collection. Plain blood is to be collected and centrifuged to get serum.

Non-treponemal tests:

RPR and VDRL are the two most commonly used non-treponemal tests.

3.4.1 Rapid Plasma Reagin Test (RPR)

The RPR card test is available as a commercial kit. It is simpler than the VDRL, and can be done even in peripheral facilities. The detailed method of doing the test is given in each kit. The main steps are as follows:

Material:

The kit contains

1. Plastic coated cards with circles on them inside which the test is performed.
2. Antigen suspension in an unbreakable container.
3. Needle 20 gauge without bevel.
4. Plastic stirrer.
5. Controls (Positive and Negative)

Other material needed but not provided with the kit:

- 1) Mechanical rotator (VDRL shaker) adjusted to rotate forming a circle of 2 cm diameter. Speed should be adjusted to the number of RPM specified in the kit.
- 2) Normal saline.
- 3) Pipettes – automatic or glass – 0.5 ml having 0.01 ml divisions, 1 ml and 2ml. Micropipettes of same capacities, with tips may also be used.
- 4) Timer
- 5) Permanent marker

Test method:

- 1) Take one test card. With the pipette place 0.05 ml of unheated serum on one of the circles.
- 2) Spread the sample on the circle with the disposable plastic stirrer.
- 3) Test known positive and negative sera along with the unknown samples.
- 4) Gently shake the RPR card test antigen and put one drop antigen in the serum sample on the circle.
- 5) Rotate the card on the mechanical rotator after adjusting the speed. The duration of the rotation should be as per the instruction with the kit.
- 6) Remove the card from the rotator and observe the result immediately with naked eye in bright light.
- 7) Reading:
Small to large clumps - Reactive
No clumps or slight roughness - Non- Reactive

If clumps occur, perform the tests in serial dilutions starting from 1 in 2, 4, 8, 16 and so on. Find out the last dilution in which clumping occurs.

3.4.2 Venereal Disease Research Laboratory Test (VDRL)

It is the standard test for syphilis which is simple, convenient, rapid and cheap.

3.4.2.1 VDRL qualitative slide test with serum**Equipment and glassware:**

- 1) VDRL rotator adjusted to 180 RPM, moving a circle of 3/4 inch diameter when kept in a horizontal position.

- 2) Microscope
- 3) Hypodermic needles cut across (without bevel) 18,19 and 23 gauges.
- 4) VDRL Slide 2 x 3 inches, with 12 ceramic rings or paraffin rings about 14 mm in diameter.
- 5) Syringe, 1 or 2 ml.
- 6) Bottles 30 ml capacity, round with glass stopper, narrow mouth, 35mm in diameter and flat inner bottom surface. Use of bottles with a convex inner bottom should be avoided, as it will not allow proper mixing of antigen.
- 7) Ring maker to make paraffin rings, 14 mm in diameter.

Reagents:

- 1) **VDRL antigen:** supplied by Institute of Serology 3, KYD Street , Kolkata should be used.
- 2) **VDRL buffered saline:** supplied by the manufacturer. It has 1% sodium chloride, pH 6.0 +/- 0.1 (Formaldehyde 0.5 ml, Na₂HPO₄ 3.037 gm, KH₂PO₄ 0.170 gm, NaCl 10.0 gm, distilled water 1000 ml).
- 3) **Normal saline (0.9%)**

Preparation of antigen emulsion:

- 1) Ideally perform the test in a temperature range of 23- 29°C (73-85°F)
- 2) Keep the buffered saline and antigen at room temperature for some time.
- 3) If temperature is low test reactivity is decreased.
- 4) If temperature is high test reactivity is increased.
- 5) Pipette 0.4 ml of buffered saline to the bottom of a 30 ml, round, glass-stoppered bottle (flat bottom).
- 6) Add 0.5 ml antigen (from lower half of 1.0 ml pipette with marking upto the tip) to the saline.
- 7) Keep rotating the bottle continuously and slowly when the antigen is added. (Note: Add the antigen drop by drop so that 0.5 ml antigen is added in 6 seconds. Pipette tip should be in the upper 1/3rd of the bottle. During rotation the outer edge of the bottle should form a circle of 2 inch diameter three times per second).
- 8) Do not touch the pipette to the saline when the last drop of antigen is added.
- 9) Rotate the bottle for 10 seconds more.
- 10) Add 4.1 ml buffered saline with a 5 ml pipette so that the total volume of formed antigen in the bottle is 5 ml.
- 11) Close the bottle and shake from top to bottom about 30 times in 10 seconds.
- 12) Leave the antigen bottle on the bench for 15-30 minutes before use. This increases the sensitivity of the antigen.
- 13) Do not mix the emulsion by taking it up with the syringe and then pushing it back to the bottle. This breaks the antigen particles and leads to a loss of reactivity.
- 14) Use the antigen within 24 hours of preparing it: after that discard any unused and remaining antigen.

Preparation and calibration of hypodermic needles for slide flocculation test:

- 1) With a file make a deep cut in the 18 gauge needle just above the tip.
- 2) Break off the needle point with pliers.
- 3) Check the needle by using a 1 or 2 ml syringe containing the antigen to be delivered. Hold the syringe with the needle perpendicular to the table top and count the number of drops falling per ml of antigen.
- 4) If too many drops fall, then the opening of the needle is too small; increase the lumen of the needle with a sharp instrument.
- 5) If very few drops per ml fall, the opening is too wide. Press the tip slightly and check the needle again.
- 6) After adjusting the needle take care not to drop it on the floor or sink or inside the bottle.
- 7) Check the needle each time it is used.
- 8) Clean the needle and syringe with water, alcohol and acetone. Remove the needle from the syringe after washing.

Testing accuracy of antigen emulsion delivery needles:

- 1) Check each day, the needle to be used. With practice the antigen and saline suspension can be dropped fast. But one should try to get drops of uniform size.
- 2) When performing the qualitative VDRL slide test on serum, the syringe should have an 18 gauge needle. The needle should drop 60 drops of antigen per ml when the syringe and needle are held vertically (upright and straight).
- 3) Use 19 gauge needle for doing quantitative test. This needle should drop 75 drops of antigen when held vertically.

Standardization of antigen suspension:

- 1) The antigen should be tested before performing the test on unknown sera.
- 2) Do the test first with known reactive, weakly reactive and non-reactive sera.
- 3) Result with these sera should be satisfactory.
- 4) If the results are not satisfactory, then do not use that antigen.

Preparation of Serum:

- 1) In a serological water bath with a temperature of 56° C, heat the specimen serum for 30 minutes.
- 2) After heating, examine the sera and again centrifuge any serum sample which shows particles.
- 3) If the test is not done for 4 hours after heating the serum, again heat the serum sample at 56° C for 10 minutes before carrying out the test.
- 4) Perform the test when the sera cool down to room temperature.

Procedure:

- 1) In summer, carry out the test early in the morning and in winter, in the afternoon so that the test is done at the prescribed room temperature of 23 - 29°C.

- 2) Using a pipette take 0.05 ml of heated serum in one ring of the ceramic ringed slide.
- 3) Glass slides with concavities, wells or glass rings should not be used for this test.
- 4) With an 18 gauge needle and syringe add 1 drop (1/60 ml) of antigen to the serum samples.
- 5) Test known positive and negative sera along with the unknown samples.
- 6) Place the slides on a VDRL rotator for 4 minutes at 180 RPM covering a diameter of 3/4 inch.
- 7) In the absence of a VDRL rotator, manual rotation may be carried out.
- 8) Keep the slide on the table and rotate it so that it makes a circle of 2 inch diameter. The speed of rotation should be 120 times per minute.
- 9) In dry climate, keep the slide in a box with lid, having a wet blotting paper. This prevents the drying of specimen during the rotation.
- 10) Read the test immediately with 10 x objective of a light microscope. It is preferable to have a simple mono ocular microscope with the mechanical stage calipers (slide holders) removed, to facilitate manual movement of slide on the stage. The antigen particles are seen as small rods. Grouping of these particles into small or large clumps defines the degree of reactivity.

Reading:

- | | | |
|---------------------------------|---|----------------------|
| Medium and Large clumps | - | Reactive |
| Small clumps | - | Weakly Reactive (WR) |
| No clumping or slight roughness | - | Non-Reactive |

3.4.2.2 VDRL quantitative tube test

Additional Equipment and Reagent

- 1) Micropipette (1000µl) with blue plastic tips.
- 2) Normal Saline (0.9 %)
- 3) Kahn tubes- 6 to 8 per reactive serum.
- 4) Rubber teats

Procedure:

- 1) Take 6 test tubes and keep them in a rack.
- 2) Pipette 0.5 ml normal saline in each tube.
- 3) Pipette 0.5 ml of test serum in tube 1 and mix well. (Serum dilution = 1:2).
- 4) Take 0.5 ml of diluted serum and add to tube 2. Mix well and transfer 0.5 ml to next tube. In this way mix well and go on adding 0.5 ml of diluted serum to each next tube till tube 6 is reached. Mix well and discard 0.5 ml of diluted serum from the last tube. The dilution obtained in the 6 tubes are 1:2, 1:4, 1:8, 1:16, 1:32, 1:64.
- 5) Take a VDRL slide and add 0.5 ml of serum from the sixth tube on ring 7.
- 6) Similarly add 0.05 ml of serum from the tube no 5, 4, 3, 2 and 1 in the wells 6, 5, 4, 3 and 2 respectively.

- 7) In well 1 take 0.05 ml of neat, undiluted serum as for the qualitative test.
- 8) Positive and negative controls for each test should be incorporated.
- 9) Add 1 drop (1/60 ml) of VDRL antigen to each ring with a 18 gauge needle and syringe.
- 10) Rotate the slides on a VDRL rotator for 4 minutes making a diameter of 3/4 inch and rotating at a speed of 180 RPM.
- 11) Observe the slide immediately under 10x magnification of light microscope.
- 12) Report the titre as the highest dilution of serum that shows a reactive result.

Reporting:

Undiluted Serum	Serum Dilution*					Report
	1:2	1:4	1:8	1:16	1:32	
(1:1)*						
R	W	N	N	N	N	Reactive undiluted only or 1 dil +
R	R	W	N	N	N	Reactive, 1:2 dilution, or 2 dils
R	R	R	W	N	N	Reactive, 1:4dilution, or 4 dils
W	W	R	R	W	N	Reactive, 1:8 dilution, or 8 dils
N (ROUGH)	W	R	R	R	N	Reactive 1:16 dilution, or 16 dils
W	N	N	N	N	N	Weakly reactive, undiluted only or 0 dil

*R = Reactive W = Weakly Reactive N = Non-reactive
 + A titre of 1:1 means that the serum was reactive in a dilution of 1 to 1. This may also be termed as "1 dil"

3.4.2.3 VDRL Quantitative slide test

Quantitative tests are performed on all reactive serum samples and on all samples showing weakly reactive or rough in the qualitative test. Successive two fold dilutions of the serum are made in 0.9 % saline and each dilution is treated as an individual serum and tested as described under qualitative test. The results are reported in terms of the highest dilution which gives a frank reactive reaction.

Additional Equipment and Reagent

1. Micropipette (1000µl) with blue plastic tips.
2. Normal Saline (0.9%)
3. VDRL slide

Procedure:

1. Pipette 0.5 ml normal saline in each well of the slide starting from well 2.
2. In well 1, take 0.05 ml of neat, undiluted serum as for the qualitative test.
3. Pipette 0.5 ml of test serum in well 2 and mix well. (Serum dilution = 1:2).
4. Take 0.5 ml of diluted serum from well 2 and add to well 3. Mix well and transfer 0.5 ml to

next well. In this way mix properly and go on adding 0.5 ml of diluted serum to each next well till well 6 is reached. Mix well and discard 0.5 ml of diluted serum from the last well. The dilutions obtained in the 6 wells are 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64.

5. Positive and negative controls for each test should be incorporated.
6. Add 1 drop (1/60 ml) of VDRL antigen to each ring with an 18 gauge needle and syringe.
7. Rotate the slide on a VDRL rotator for 4 min. making a diameter of 3/4 inch and rotating at a speed of 180 RPM.
8. Observe the slide immediately under 10 x magnification of light microscope.
9. Report the titre as the highest dilution of serum that shows a reactive result.

NOTE: The quantitative slide test for RPR test is also to be performed as described for VDRL, but by using the RPR antigen.

3.4.2.4 VDRL qualitative slide test on Cerebro Spinal Fluid (CSF):

In suspected neurosyphilis, VDRL should be done in CSF sample. **This is the only non-treponemal test that can be done in CSF.**

Additional equipment and reagents:

1. Hypodermic needle cut across (without bevel) 21 or 22 gauge.
2. Saline 10% (10gm dry sodium chloride in 100 ml of distilled water).
3. VDRL slides, 2 x 3 inch in size, with concavities measuring 16 mm in diameter and 1.75 mm in depth.

Preparation of "Sensitized antigen suspension":

1. Prepare the antigen as described for VDRL slide test.
2. Mix 1 part 10 % saline and 1 part VDRL slide test antigen.
3. Mix by slowly rotating the bottle and keep it on bench for 5 minutes before use. Do not use the antigen till 24 hours after preparation.

Testing of delivery needles:

1. Check needle each time before use.
2. For both qualitative and quantitative test on CSF, drop the sensitized antigen from a syringe with 21 or 22 gauge needle that delivers 100 drops of antigen suspension per ml when syringe and needle are held vertically.

Preparation of cerebrospinal fluid:

1. Centrifuge the spinal fluid. Remove clear upper part of the CSF into another tube with a Pasteur pipette.
2. Do not heat spinal fluid (different from serum, which is heated)
3. Do not test any turbid CSF or CSF with visible blood.

Procedure:

1. Take 0.05 ml spinal fluid in one concavity of the VDRL slide.
2. Put 1 drop (0.01 ml) of the sensitized antigen emulsion in the spinal fluid sample with 21 or 22 gauge needle.
3. Rotate the slide on VDRL rotator for 8 min. at 180 RPM speed.
4. Read the test immediately with 10x objective of a light microscope.

Reading:

- Visible clumping - Reactive (R)
No clumping or very slight roughness - Non-reactive (NR)

3.4.2.5 VDRL Quantitative tube test on spinal fluid:

1. Perform quantitative test on all CSF samples found to be reactive by qualitative test.
2. Prepare spinal fluid dilution as follows:
3. Take 0.2 ml of 0.9% saline in five or more test tubes.
4. Pipette 0.2 ml of spinal fluid (unheated) in tube 1 and mix well.
5. Take 0.2 ml from tube and add to tube 2.
6. Mix and transfer diluted CSF from each tube to its next tube till the last tube is reached. Discard 0.2 ml of diluted spinal fluid from the last tube. The dilutions obtained will be 1:2, 1:4, 1:8, 1:16, 1:32 and so on.
7. Perform the test as described for "VDRL qualitative test for spinal fluid".
8. Report highest dilution of the cerebrospinal fluid giving reactive results as the titre.

Limitations of Non-treponemal test

VDRL test has a limitation of Biological False Positive (BFP) reactions and Prozone phenomena.

Biological False Positive (BFP) reactions are defined as positive reaction in a cardiolipin test and a negative reaction in a confirmatory treponemal test. Quantitative RPR/VDRL test with a titre $\geq 1:8$ is considered as significant titre and is suggestive of syphilis. It is recommended that a confirmatory test such as TPHA should be performed on all sera with a reactive RPR/VDRL regardless of its titre especially to accurately diagnose and confirm syphilis in case having titres $<1:8$. Some of the TPHA positive cases with RPR/VDRL titre $<1:8$ may be either because of biological false positivity or representative of early / latent / late syphilis cases or treated cases of syphilis. In addition, combination of this testing strategy (RPR/VDRL + TPHA) is less expensive and much easier to perform. BFP reactions can be observed in various acute and chronic conditions in the absence of syphilis. These can be acute, giving positive reaction for few weeks to few months or chronic positive RPR/VDRL reaction lasting more than six months. Examples of BFP reactions are; infections, injuries, inflammation and early HIV infection, SLE, collagen vascular diseases, leprosy, malaria relapsing fever, hepatitis, Infectious mononucleosis, tropical eosinophilia etc.

Prozone phenomena is a negative reaction in a test due to excess amount of antibody. The serum must be serially diluted to detect prozone phenomena. Screening undiluted specimens with a non-treponemal test alone can yield false negative reactions in the presence of high titres of antibody (the prozone phenomenon), for example in secondary syphilis.

3.5 Treponemal tests

All serum samples which are reactive or weakly reactive should be tested by a treponemal test (e.g. TPHA, FTA-Abs) for confirmation. Treponemal tests are specific and once positive; remain so even after successful treatment. In order to monitor response to treatment, non-treponemal tests are used.

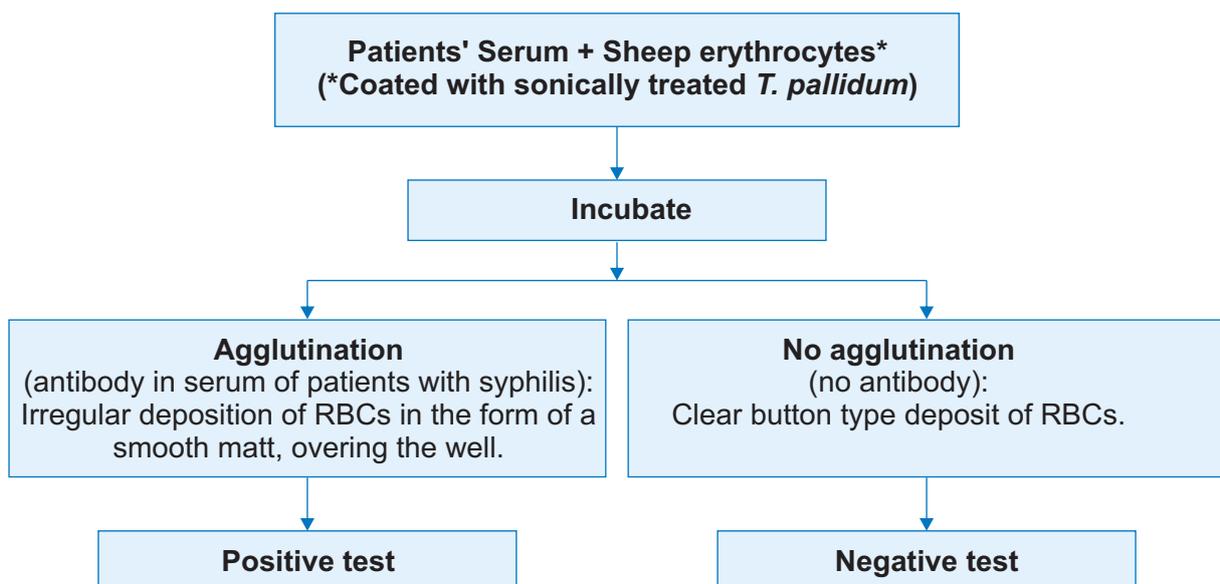
3.5.1 *Treponema pallidum* haemagglutination assay (TPHA):

It is one of the confirmatory tests for the diagnosis of syphilis and is done in microtitre plates.

In this test, red blood cells coated with *T.pallidum* are mixed with the serum of patients. If antibodies to *T.pallidum* are present the red cells will agglutinate in the form of a smooth matt at the bottom of the microtitre plate well. If no antibodies are present then no agglutination occurs and the red cells settle at the bottom of the well in button form.

Many TPHA kits are available commercially. Test procedures are different for each kit. For detailed information see the instructions provided with the kit.

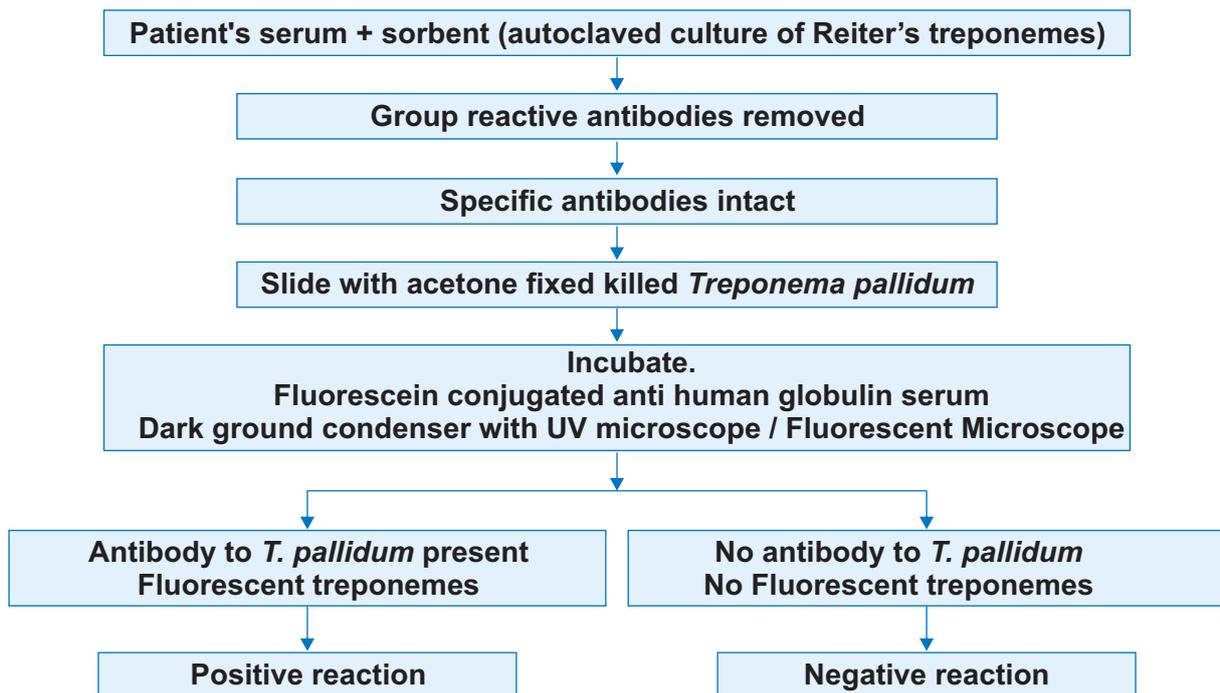
The principle of TPHA is;



3.5.2 Fluorescent Treponemal Antibody Absorption (FTA-Abs) Test:

It is a confirmatory test for syphilis. Always follow the instructions provided with the kit for

Principle of FTA-Abs is:



3.6 Point of care/Rapid tests for syphilis.

World over, the traditional testing for syphilis has consisted of initial screening with an inexpensive non-treponemal test (usually VDRL or RPR), then retesting reactive specimen with a more specific, and expensive, treponemal test (like TPHA, TPPA, EIA, FTA-Abs). When both test results are reactive, they indicate present or past infection. However, in recent times, there has been a reversal of this sequence, wherein some laboratories begin with a rapid, treponemal test like EIA or ICS (Immuno chromatographic strip test) as the initial testing procedure and then retesting reactive results with a non-treponemal test. This approach has introduced complexities in the test interpretation that did not exist with the traditional sequence.

An important consequence of this reversal is the identification of a combination of reactive and non-reactive test results that would not otherwise have been identified. Screening with a treponemal test sometimes identifies persons who were reactive to the treponemal test but non-reactive to the non-treponemal test. Consequently, use of a reversed sequence of syphilis testing might result in over diagnosis and over treatment of syphilis in some clinical settings.

According to some studies when results are reactive to the treponemal test but non-reactive to the RPR test, persons with history of previous treatment will require no further management. For person without the history of treatment, a second, different treponemal test should be performed. If the second treponemal test is non-reactive, the clinician may decide that no further evaluation or treatment is indicated, or may choose to perform a third treponemal test to resolve the discrepancy.

Several rapid tests for syphilis are now commercially available. These are simple point of care tests that can be performed outside a laboratory setting with minimal training and no

equipment, using only a small amount of whole blood collected by a finger prick. The result is easy to interpret and available in 30 minutes. Most rapid syphilis tests are made in a dipstick or cassette format. Hence they can address the problem associated with lack of access to a laboratory and the low patient return and can be used in all health care settings to allow immediate treatment. But, results of the rapid test are reproducible only if the tests are stored and performed according to manufacturer's instructions, including special attention to expiry dates.

Thus, given the anticipated increase in use of treponemal tests for screening, the introduction of rapid tests in a country or a specific area, should only be undertaken after due consideration to the following aspects- Test performance, ease of use, format of the testing, shelf life and temperature stability, requirement of additional supplies for testing, access to the test, quality of testing, availability of treatment to sero reactive cases and cost. Further, the epidemiology of disease in various countries, the different patterns of sero reactivity and systems of health care have to be ascertained.

Hence, countries that have already established effective syphilis control programmes, including screening for antenatal and high risk populations, should preferably maintain their programme rather than introduce rapid tests.

3.7 Nucleic acid testing

A number of PCR-based methods such as real-time, in-house and multiplex PCRs (for detection of Herpes simplex virus *T.pallidum*, *Haemophilus ducreyi*) have been developed for the detection of *T.pallidum* in clinical specimens. These methods have been found to be highly sensitive, able to detect as low as one to 10 organisms per specimen with high specificity. These methods are also the most practical in certain settings. PCR undoubtedly holds promise as a test of choice for congenital syphilis, neurosyphilis and early primary syphilis when traditional tests have limited sensitivity. This method could be used to monitor treatment, and there is also potential to use it to differentiate new infections from old infections. However, serology remains the mainstay for diagnosis of syphilis because of its ease and cost-effectiveness in resource-poor settings.

SUMMARY

1. Immediate diagnosis of *T.pallidum* infection by dark field microscopy makes it a very useful diagnostic tool. So this test should be done in all laboratories at the intermediate and tertiary centres.
2. A simple and quick serological test like rapid plasma reagin (RPR) card test should be done at the peripheral level.
3. VDRL test is the test to be done at the intermediate and central levels. Quantitative VDRL test should be done to find the actual titre of the antibody. VDRL on CSF must be done to diagnose neurosyphilis.
4. In a central laboratory a confirmatory test like TPHA should be done. With due consideration to cost constraints, if available, test may be done at the intermediate level also.
5. The Fluorescent treponemal antibody absorption (FTA-Abs) test is very specific and helps in early diagnosis of syphilis. It is better than haem-agglutination assay. Experienced and highly trained staff are needed to do the test, which is also time consuming. Because the test is costly, it is done only to confirm the result of other tests at the central laboratory.

4. CHANCROID

4.1 *Haemophilus ducreyi*

Haemophilus ducreyi is the causative agent of chancroid which is a genital ulcerative disease. This disease occurs as multiple painful genital ulcers, which bleed easily. Painful enlargement of the inguinal lymph nodes on one side only may be seen. Abscess may form in the lymphnode and this is called a bubo. *H. ducreyi*, a Gram-negative coccobacillus, is a fastidious organism and is difficult to grow without specialised media.

4.2. Tests that can be done to diagnose infection due to *H. ducreyi* are:

Sample	Test
Swab from ulcer, Aspirate from ulcer/bubo	Gram staining and microscopy
Swab from ulcer, Aspirate from ulcer/bubo	Culture, β -lactamase testing and antibiotic sensitivity

4.3. Gram staining and microscopy:

Materials required:

1. Dry, sterile gauze
2. Sterile swab
3. 0.85% sterile saline
4. Clean, grease free microscope slides
5. Gram's stain with rack
6. Binocular bright field microscope
7. Glass marking pencil / permanent marker
8. 70% Alcohol and povidone iodine to clean skin
9. Syringe and needle

Method:

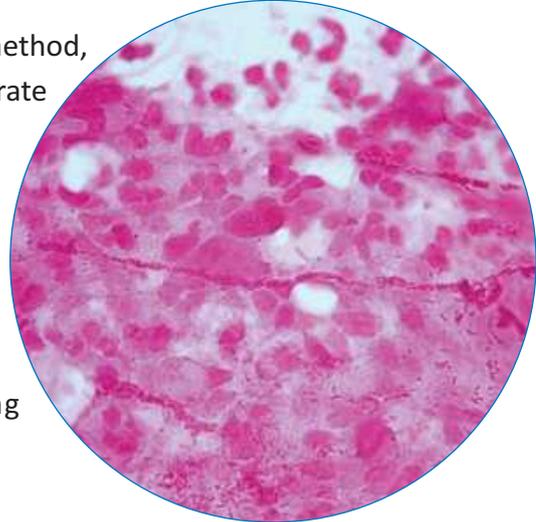
1. Ulcer: Clean the ulcer with sterile swab moistened with saline to remove the dry crust.
2. Collect the exudates from the ulcer base with a sterile swab moistened with saline by rubbing vigorously at the base of the lesion.
3. Abscess: Disinfect skin with alcohol and iodine. Aspirate fluid with a needle and syringe.
4. Prepare smear on a clean, grease free microscope slide by rolling the swab stick 180° in one direction. This preserves the arrangement of the organisms.
5. Stain by Gram's method.
6. Observe under oil immersion lens of light microscope.

Reading:

Gram negative coccobacilli, 1-2µm in size with rounded ends, slender or short, plump rods. Bacteria are arranged in long parallel rows ('school of fish'), short chains or clusters. Bacteria may be inside or outside the cells.

Figure 3 - Gram stain of *H. ducreyi*

Only about 50% of the cases can be detected by this method, therefore, culture is necessary in most cases for accurate diagnosis.

**4.4. Culture for *H. ducreyi*****Materials required:**

1. Enriched Gonococcus agar (Annexure-II) and enriched Mueller Hinton agar (Annexure-II)
2. Desiccator (candle jar)/ CO₂ Generating Gaspack/CO₂ Incubator
3. Incubator set at 33°C.
4. Candle (White colour wax)
5. Platinum loop
6. Moist cotton
7. Bunsen burner or spirit lamp
8. Glass marking pencil / permanent marker

Method:

1. It is advisable to inoculate two different selective agar plates at the point of sample collection itself.
2. Inoculate the material on both isolation media and send to the laboratory immediately for incubation.
3. If sample is transported from a primary level laboratory, transport swabs/aspirates in Amies transport medium (Annexure-II) . Transport at 4°C and make sure that the sample reaches the culture laboratory within three days.
4. Keep the inoculated media in a candle jar with moist chamber and incubate at 32 -34°C.
5. Read the cultures after 48 hours incubation.
6. If negative after 48 hours, keep up to 5 days before finally calling it negative.

Reading of cultures:

Small colonies up to 0.5 mm in diameter.

Greyish yellow, granular, raised non-mucoid colonies, translucent to opaque.

Typically, the colonies move intact on the surface of the media when pushed with a platinum loop.

Difficult to pick up the colony from the media.

Identification of *Haemophilus ducreyi*: Perform the following tests from a young culture and determine if the reactions agree with those listed below.

a. Smear:

Gram staining (Annexure-I)

- ▶ Gram negative coccobacilli.
- ▶ Arranged in short chains, whorls or clumps.
- ▶ Organism staining at the two ends only (bipolar staining) is seen.
- ▶ More than half the organism show different distinct forms (Pleomorphism).

b. Catalase negative

c. Oxidase Test: positive (Refer *N. gonorrhoeae* for details) May be a little slow.

d. Indole negative

e. ALA Test: Negative (Aminolevulanic Acid test for porphyrin synthesis)

f. X factor & V factor requirement: *H. ducreyi* requires factor X for growth but is factor V independent.

g. Nitrate Reduction Test: Positive

- ▶ Prepare a suspension of 8-10 colonies in 0.5 ml of saline.
- ▶ Take 0.04 ml of this suspension in a sterile test tube.
- ▶ Add 0.04 ml of 0.05 % NaNO₃ and 0.04 ml of 0.025M phosphate buffer (Annexure-II) with a pH of 6.8 in the tube.
- ▶ Incubate the tube in a water bath at 37°C for 1 hour.
- ▶ Add 0.06 ml of 0.8% sulphanilic acid in 5N acetic acid and 0.06 ml of 0.5 % α -naphthylamine in 5N acetic acid to the bacterial mixture.
- ▶ Shake the tube and read.

Interpretation: Development of pink colour-positive test.

g. Test with SPS disc (Sodium Polyanethol Sulphate) on Chocolate Agar. *H. ducreyi* is sensitive with a zone of inhibition of more than 10 mm.

4.5 β -lactamase test

- 1) In some geographical regions many isolates of *H. ducreyi* form β -Lactamase.
- 2) β -Lactamase test here helps in guiding correct treatment. (refer to *N. gonorrhoeae* for details)

4.6 Antimicrobial Susceptibility testing

1. Many *H. ducreyi* isolates are resistant to most of the commonly used antibiotics. So antimicrobial susceptibility testing is necessary. But this test in *H. ducreyi* is difficult. So it is done only in selected reference laboratories.
2. Kirby Bauer disc diffusion technique is not an approved method. Agar dilution or broth dilution method should be used for determining antimicrobial susceptibility.
3. Antibiotics tested are Tetracycline, Chloramphenicol, Streptomycin, Ceftriaxone, erythromycin and Kanamycin.

SUMMARY

1. Direct smear sensitivity is less in the diagnosis of *H. ducreyi* infection.
2. Culture is necessary for diagnosis, but it is very difficult to culture *H. ducreyi*.
3. Facilities for culture and antimicrobial susceptibility testing should be available at the central level.

5. GRANULOMA INGUINALE (*DONOVANOSIS*)

5.1. *Klebsiella granulomatis*

Granuloma inguinale is a bacterial disease caused by *Klebsiella granulomatis* characterized by ulcerative genital lesions. Small, painless nodules appear after about 1-12 weeks. The lesion may be nodular, ulcerovegetative (most common), cicatricial, hypertrophic or verrucous (relatively rare). The most common locations of granuloma inguinale lesions in men are the sulcocranal and balanopreputial regions, as well as the anus. In women, granuloma inguinale lesions occur on the labia minora, the mons veneris, the fourchette, and/or the cervix. Cervical involvement occurs in 10% of cases. Spread to inguinal region gives rise to formation of pseudobubo.

5.2. Tests done

Sample	Test
Tissue from ulcer	Giemsa staining and microscopy

5.3. Materials required:

1. Sterile gauze.
2. Sterile saline.
3. Thin scalpel or punch biopsy forceps.
4. Grease free, microscope slides.
5. Giemsa stain.
6. Glass marking pencil / permanent marker

5.4. Procedure

Clean an area of granulomatous tissue in the red lesion preferably near the edge with a gauze soaked in saline and finally with a dry gauze.

5.4.1 Preparation of Smear

- a. Direct impression smear
 - ▶ Make direct impression smears from the lesion on a glass slide
 - ▶ It is usually not adequate because surface debris and other bacteria are liable to obscure the picture.
- b. From Tissue
 - ▶ Using a blunt scalpel or a punch biopsy forceps remove a small piece of tissue
 - ▶ Make an impression smear from the moist surface of the tissue
 - ▶ Alternatively tissue can be crushed between two slides.
- c. Swab
 - ▶ A cotton swab can be rolled over the surface of the lesion
 - ▶ Make smears immediately with moist tissue as any drying renders the smear more

difficult to make and, if excessive drying occurs while making a smear, cells containing Donovan bodies tend to rupture.

- ▶ Once the smear has been made it can then be air dried and heat fixed before staining. Other forms of fixation may shrink the Donovan bodies.

d. Tissue Biopsy

- ▶ Biopsy is recommended for lesions in which organisms are likely to be scarce, that is, very early or very sclerotic lesions and those with heavy superinfection.
- ▶ Biopsy is mandatory in cases where malignancy is thought possible or antibiotic therapy fails to give improvement.
- ▶ Collect the tissue as described above.

5.4.2 Prepared smears can be examined microscopically under oil immersion lens of a light microscope after staining.

- ▶ Giemsa, Leishman or Wright's stain are all satisfactory.
- ▶ For rapid staining, Rapi Diff stain can be used.
- ▶ For fixed or embedded tissue sections, Giemsa or silver stains are preferred, haematoxylin and eosin is not satisfactory

Giemsa Stain Procedure:

- ▶ Fix the smear in pure methanol for 30 seconds, by immersing it or by putting a few drops of methanol on the slide.
- ▶ Stain with 1 part stain and 9 part buffer for 30 minutes then flush with tap water and leave to dry. (Annexure-1)

RapiDiff Stain

- ▶ Place the smear in RapiDiff fixative for 15 seconds (6 dips)
- ▶ Then place it in solution RapiDiff 1 (Eosin Y) for 15 seconds (6 dips)
- ▶ Place in solution RapiDiff 2 (Thiazine Dye Mixture) for 15 seconds (6 dips)
- ▶ Finally rinse the slide in phosphate buffer pH 6.8.
- ▶ Air dry the stained slides and examine by light microscopy under oil immersion (1000 x magnification). (Annexure-1)

5.5. Reading and Interpretation

- ▶ Many mononuclear cells with cystic spaces, with or without organisms.
- ▶ The mononuclear cell may be between 20-90 μm in size.
- ▶ Cytoplasm of the mononuclear cells shows cystic spaces of 1-10 μm size.
- ▶ Inside the cystic spaces are the blue-purple coloured coccobacilli, usually capsulate, short plump rods 1 – 1.5 μm in length and 1 μm in breadth. Capsule stains pink.
- ▶ The organisms may be curved or dumb-bell shaped.

- ▶ Usually show staining at the two ends and so resemble a closed safety pin ('Safety pin appearance').
- ▶ Donovan bodies: diagnostic of this infection, appear as coccobacilli inside large vacuoles (giving the appearance of being surrounded by a halo) in the cytoplasm of the large histiocytes, plasma cells and polymorphonuclear leucocytes.
- ▶ Upto 25 organisms may be seen inside a single macrophage.

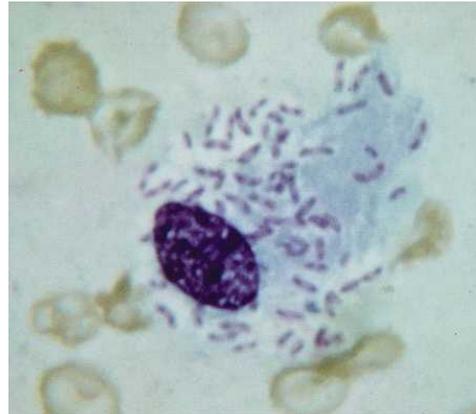
Culture

K. granulomatis does not grow on any standard microbiological laboratory media

Others tests

- ▶ Polymerase chain reaction techniques may be more sensitive; however, they are currently only used for scientific research.
- ▶ An indirect immunofluorescent technique is available to test serum; however, it is not accurate enough for confirmatory diagnosis.
- ▶ Papanicolaou smears may identify Donovan bodies in patients undergoing routine cervical cytological screening.
- ▶ If bony involvement is suspected in granuloma inguinale, radiography or other imaging studies are indicated
- ▶ Testing for other sexually transmitted diseases is warranted because multiple coexisting infections are common.

**Figure 4 -
Giemsa Stain of *K. granulomatis***



Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine*, 17th Edition: <http://www.accessmedicine.com> Copyright © The McGraw-Hill Companies, Inc. All rights reserved.

SUMMARY

1. Direct microscopy is performed by impression smears or crushed tissue between two slides and staining by Giemsa stain.
2. *K. granulomatis* does not grow on any standard microbiological media in the lab.

6. BACTERIAL VAGINOSIS

Bacterial vaginosis (BV) is a clinical syndrome of unknown etiology characterized by an overgrowth of altered vaginal flora (usually polymicrobial) and variable degrees of depletion of normal *Lactobacillus* sp. population.

The altered vaginal flora is characterized by the presence of the following bacteria:

- ▶ *Gardnerella vaginalis*
- ▶ *Mobiluncus mulieris*
- ▶ *Mobiluncus curtissii*
- ▶ *Bacteroides* sp.
- ▶ *Peptostreptococcus* sp.
- ▶ *Prevotella* sp.

The patient comes with the symptoms of foul smelling (due to the release of amines produced by anaerobic bacteria that decarboxylase amino acids like lysine and arginine), fishy vaginal odour. Grey homogeneous, thin vaginal secretion at the vaginal opening is usually seen.

6.1. Collection of specimen

Method:

1. Collect the specimen with gloved hands and vaginal speculum in place.
2. Collect the specimen from the lateral or posterior fornix of vagina with a sterile cotton wool swab soaked in normal saline.

6.2. Procedures

A. Vaginal pH test:

1. Take pH indicator paper strips with a range of ± 3.8 to ± 6.0 .
2. Touch the specimen swab on the pH paper, or
3. Touch the pH paper to the tip of the vaginal speculum after removing it from the vagina or touch the pH paper to the wall of vagina directly.
4. Do not allow the contact of pH paper with cervical secretions.

Reading:

- ▶ Normal adult vagina has an acid pH of 3.6 to 4.2
- ▶ In Bacterial vaginosis the pH is raised to 4.5 or more.

NB. Presence of menstrual blood, cervical mucus, semen or *T. vaginalis* infection may also raise the vaginal pH.

B. Amine test (Whiff test):

1. Take a drop of vaginal fluid on a clean grease free microscope slide.
2. Put a drop of 10% potassium hydroxide (KOH) on the vaginal fluid. On addition of KOH, amine becomes volatile producing fishy odour.
3. Bring the slide close to the nose to smell the amine odour.
 - An intense putrid fishy odour indicates a positive reaction.
 - The secretion becomes odourless soon after the test.

Note: The test is subjective. Absent in 50% of individuals.

C. Microscopy

1. Wet mount examination:

- a. Take a drop of vaginal fluid on a clean grease free microscope slide and mix with a drop of normal saline. Alternatively take a drop of normal saline on a clean grease free microscope slide and gently rub the swab over it to mix the vaginal fluid with it.
- b. Put a coverslip on the mixture and see to it that the preparation is free of air bubbles and uniformly spread.
- c. Observe the slide under 40 x magnification of the objective.

Reading:

Presence of "Clue cells" (> 20%) suggests the diagnosis.

A clue cell is a squamous epithelial cell with many coccobacillary organisms attached to its surface giving it a granular appearance. The cells do not have a well defined edge because of the presence of the bacteria and disintegration of the cell. Over 20 % clue cells are seen along with the normal vaginal epithelial cells in a case of bacterial vaginosis. In most of the cases the organism attached to the epithelial cells is *G. vaginalis*.

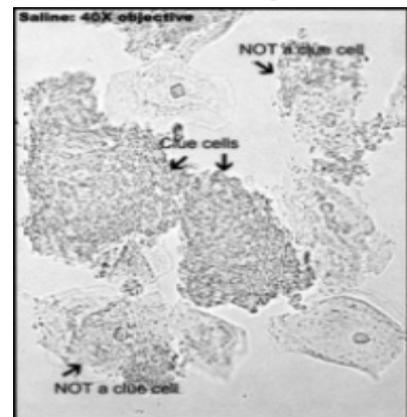
2. Gram Stained Smear:

- a. Prepare a smear on clean grease free microscope slide by rolling the swab on the slide.
- b. Stain the smear with Gram stain after drying and heat fixing.
- c. Observe the smear under oil immersion lens after placing a drop of liquid paraffin on the slide.

D. Amsel's Criteria (Clinical Criteria)

1. Thin, homogenous, adherent discharge
2. Elevated pH >4.5 (normal 3.6-4.2)
3. Fishy odor on addition of 10% KOH (Whiff test or amine test)
4. Clue cells (>20%)
(Any three criteria should be satisfied)

**Figure 5 -
Wet mount showing clue cells**



E. Nugent Scoring:

The Nugent scoring system for Gram stain is a weighted combination of lactobacilli, *G. vaginalis* or Bacteroides (small Gram-variable or Gram-negative rods) and curved Gram-variable rods (Mobiluncus). This standardized 0-10 scoring system is presented in table below. Each morphotype is quantitated from 1 to 4 + with regard to the number of morphotypes per oil immersion field. The sum of the weighted quantitations of the three morphotypes yield a score of 0 to 10.

Lactobacillus morphotypes- Large sometimes long Gram positive rods

Gardnerella/Bacteroides morphotypes-Small Gram variable/Gram negative rods

Mobiluncus morphotypes-Curved Gram variable/ Gram negative rods

At least 5 oil immersion field (OIF) should be examined to determine an average of each morphotype. Total score = Lactobacilli + *G.vaginalis* and Bacteroides spp. + Gram variable rods.

Nugent's scoring system for Gram-stained vaginal smears

Lactobaacillus	Score	Gardnerella, Bacteriodes	Score	Curved Gram -ve bacilli	Score
30 or >	0	0	0	0	0
5-30	1	<1	1	<1	1
1-4	2	1-4	2	1-4	1
<1	3	5-30	3	5-30	2
0	4	30 or >	4	30 or >	2

Interpretation:

score	Report
0-3	Negative for Bacterial Vaginosis
4-6	Inter mediate
7-10	Positive for Bacterial Vaginosis.

To conclude, a stained smear gives a better idea about the normal vaginal organisms and the identification of 'Clue cells' is better by Gram-stained method as compared to wet preparation.

6.3. Culture

G. vaginalis can be cultured on human blood bilayer plate. Culture, however, is not recommended for diagnosis.

SUMMARY

1. Wet mount examination and the pH test can be done at the peripheral level for bacterial vaginosis.
2. The pH test, KOH test, wet mount examination and Gram staining can all be done at the intermediate and central level.
3. Using the Nugent's criteria, the sensitivity of Gram stain for diagnosis of BV is very high.

7. CANDIDIASIS

7.1 *Candida* species

Vulvovaginal candidiasis (VVC) is caused by the fungus *Candida albicans* in approximately 85% of cases, with *C. glabrata* being responsible for the remaining 15%. Other species, such as *C. krusei*, and *C. tropicalis* rarely cause vaginitis. *Candida* species are usually of endogenous origin and can be isolated from the genital tract in up to 25% of asymptomatic healthy women of childbearing age. To colonize the vagina, *Candida* species must first adhere to the vaginal epithelial cells and then grow, proliferate, and germinate, before finally causing symptomatic inflammation. Important predisposing factors for colonization and inflammation include: changes in reproductive hormone levels associated with premenstrual periods, pregnancy, and oral contraceptives, the use of antibiotics, diabetes melitus, and immunosuppression. Diagnosis consists of demonstration of *Candida* in vaginal secretions in symptomatic women.

Since *Candida* is part of normal flora, serological tests or nucleic acid hybridisation or amplification tests do not play a diagnostic role as they can lead to false-positive results.

7.2 Collection of specimen;

Materials required:

1. Sterile cotton wool swabs.
2. Sterile saline.
3. Vaginal speculum.
4. Spirit lamp.
5. Clean, grease free microscope slides.
6. 10% KOH
7. Clean, grease free glass cover slips.
8. Sabouraud's dextrose agar (SDA) slants in test tubes.
9. Incubator set at 25 – 30°C
10. Immersion oil
11. Inoculating loop
12. Glass marking pencil / permanent marker

In Females:

1. Collect scrapings from vagina and edges of erythematous (red) lesions of the vulva, with sterile cotton wool swabs soaked in saline.
2. Collect pooled vaginal discharge from the posterior fornix in a sterile container.
3. Collect urethral swab if necessary.

In Males:

Swab from the sub-preputial (area under prepuce) area and urethra is collected, if necessary.

Transport of specimens;

Put the swab in a sterile bottle containing some saline and send to the laboratory immediately. If there is some delay, the sample should be kept in the fridge at 4°C.

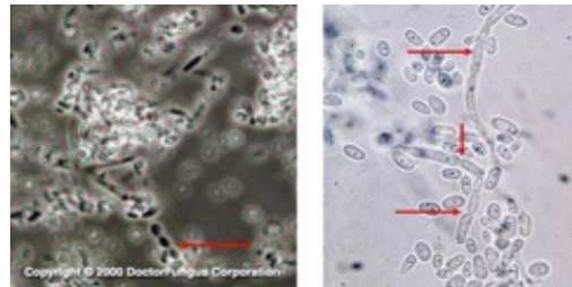
7.3 Microscopy

KOH Wet mount :

1. Place the specimen on a clean, grease free microscope slide.
2. Mix 2 drops of 10% potassium hydroxide (KOH, 10 gram/100ml) with the specimen.
3. Put a clean cover slip over the specimen.
4. See that no air bubble is trapped under the cover slip.
5. Gently heat the slide over a flame for 10-20 sec. But do not allow to boil.
6. Observe the slide under 40x objective of light microscope.
7. Saline wet mount can also be used but adding KOH makes it a better test.
8. About 50-80% of patients can be diagnosed by this method.

Note: The KOH must be prepared fresh every two weeks or whenever crystals are seen in microscopy.

**Figure 6 -
Wet mount showing budding yeast cells**



Reading:

Round or oval budding yeast cells, about 5 - 7µm in diameter – Presence of mycelia or pseudohyphae.

Gram Staining:

1. Prepare a smear on a clean, grease free microscope slide.
2. Heat fix the slide (Annexure-I).
3. Stain the slide by Gram's method (Annexure-I).
4. Observe under oil immersion lens of light microscope.

Reading:

Gram positive (violet) budding yeast cells (looking like figure of 8) and yeast hyphae.

7.4 Culture for Candida

Culture is necessary for isolating the fungus in pure form, and for species identification of Candida. As a diagnostic procedure it should be done whenever candidiasis is clinically suspected.

1. Inoculate the sample on two Sabouraud's dextrose agar with chloramphenicol slopes (Annexure-II).
2. Incubate one slope at room temperature (25-27°C).
3. Incubation of other slope at 37°C gives faster growth.
Pasty, opaque, cream coloured colonies grow in 24-48 hours.

7.5 Identification of Candida Species

A. Saline wet mount :

1. Take 2 drops of normal saline on a clean, grease free microscope slide.
2. Pick up one colony with a sterile platinum loop and make a uniform emulsion of the colony.
3. Place a coverslip over the emulsion and observe under 40 x magnification of light microscope.
4. Presence of budding yeast cells confirms the diagnosis.

B. Germ tube test:

1. The test is done to confirm the diagnosis of *C. albicans*.
2. The test is positive in over 98% of isolates.
3. Inoculate a few colonies into 0.5 ml of pooled human serum.
4. Incubate at 37°C for 2 hours in a waterbath or incubator.
5. After 2 hours take 2 drops of suspension on a clean, grease free microscope slide and mount with a coverslip.
6. Observe under 40 x objective magnification of light microscope.

Reading:

Observation of germ tube confirms the presence of *C. albicans*. A germ tube is a short, lateral hyphal extension (filament) of the yeast cell and is not constricted at the base.

C. Chlamydospore and blastospore formation:

1. This test is yet another method for confirmation of *C. albicans*, as most isolates form Chlamydospores and blastospores.
2. Take a plate of cornmeal Tween agar (CMA) (Annexure-II). On one half inoculate a test strain and on the other half inoculate a control strain of *C. albicans* (ATCC 90028).
3. With a sterilized straight wire pick up few colonies of Candida from culture and cut into the agar as parallel lines about 1 cm apart and then criss- cross several times.
4. Pick a cover slip with a forceps, heat it over the flame and allow it to cool.
5. Place it over the surface pattern and incubate the plate at 25 - 30 °C for 18 to 48 hours.
6. Observe under 40 x magnification of a light microscope.

Reading:

Hyphae and pseudohyphae with clusters of blastospores (4-5µm) at internodes. Terminal chlamydospores, 8-12µm. Formation of Chlamydospores is characteristic of *C. albicans*. Occasionally *C. stellatoidea* may also form chlamydospores. Further confirmation is then done by carbohydrate assimilation test.

D. Carbohydrate assimilation test:

1. Take a sterile Petri dish and divide the plate into four quadrants by marking the back of the plate with a glass marking pencil.
2. Melt the basal medium agar (Annexure-II) and pour about 20 ml in the plate
3. Prepare 2ml of pure yeast colonies suspension (approx. 10^6 colonies/ml).
4. Label each quadrant of the plate with the appropriate initial of the carbohydrate to be used.
5. With the help of a sterile cotton wool swab inoculate the agar surface with the yeast suspension.
6. Drop the appropriate carbohydrate solution (Annexure-II) on each quadrant with a separate dropper.
7. Incubate the plate at 25 - 30°C for 24 to 72 hours.

Reading:

A growth ring of the yeast and a change in indicator colour from purple to yellow – positive test. Carbohydrate is assimilated.

No growth and no change in the indicator colour – Negative test. The isolate lacks enzymes to assimilate that particular carbohydrate.

For carbohydrate assimilation reactions of *C. albicans* and *C. stellatoidea* refer table below:

Table. Carbohydrate assimilation reactions of

***C. albicans* and *C. stellatoidea*.**

Carbohydrate	Organism	
	<i>Candida albicans</i>	<i>Candida stellatoidea</i>
Dextrose	+	+
Maltose	+	+
Sucrose	+	-
Lactose	-	-
Galactose	+	+
Trehalose	+	+
Raffinose	-	-
Cellobiose	-	-
Melibiose	-	-
Inositol	-	-
Xylose	+	+

E. Use of Chromogenic medium for identification of candida is recommended at intermediate level laboratories which may find it difficult to perform carbohydrate assimilation tests for identification of candida. Candida of different species show different colours and colony morphology based on which they are speciated as follows:

Appearance of Candida on Chromogenic media

Species	Color	Morphology
<i>C. dubliniensis</i>	Green	--
<i>C. famata</i>	Pink to lavender	–
<i>C. firmetaria</i>	Pink to lavender, pale border	Some flat, rough; others waxy
<i>C. glabrata</i>	Cream or ivory colour	Small to medium, smooth, convex, creamy
<i>C. guilliermondii</i>	Pink to lavender	–
<i>C. inconspicua</i>	Pink to lavender, pale borders	Flat, rough
<i>C. kefyr</i>	Pink to lavender, often with darkened centers	Large, rough
<i>C. lipolytica</i>	Ivory to pink	Large, flat, rough, wrinkled
<i>C. lusitaniae</i>	Pink to lavender	Some waxy
<i>C. norvegensis</i>	Ivory to pink	Large, rough
<i>C. parapsilosis</i>	Ivory to pink to lavender	Small to medium, smooth to wrinkled
<i>C. rugosa</i>	Light blue-green, pale border ^e	Medium to large, flat
<i>C. albicans</i>	Green	Large smooth colony
<i>C. krusei</i>	Pink, pale borders	Medium to large, flat, rough, dry and wrikled
<i>C. tropicalis</i>	Steel blue, purple diffusion	Medium smooth colony

7.6. Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing is not recommended as a routine for vaginal candidiasis. Reference laboratories should test candida isolates against Clotrimazole, Nystatin and Fluconazole using MIC determination by agar or broth dilution method with an objective to evaluate treatment protocols. E test strips are also available for anti fungal drug susceptibility testing.

SUMMARY

1. Potassium hydroxide wet mount examination should be done at the peripheral level for suspected Candida spp. infection.
2. Gram stain may also be performed at the peripheral level for suspected Candida infections.
3. Confirmation of Candida infection by culture can be done at the intermediate and central level laboratories. Culture should only be done when candidiasis is clinically suspected while the wet mount is negative.
4. Speciation of Candida can be done at the intermediate or central level. This requires culture of the organism.
5. There is no indication for routine susceptibility testing for candida. It may be done at the reference laboratories to review treatment recommendations from time to time

8. TRICHOMONIASIS

Trichomonas vaginalis is an anaerobic protozoan parasite. It is transmitted sexually. In most infected women and men, the infection shows no symptoms.

8.1 Importance of laboratory diagnosis of trichomoniasis

Patients suspected of having trichomoniasis generally presents with

- ▶ Yellowish green frothy discharge
- ▶ Pruritus
- ▶ Dysuria
- ▶ Dyspareunia
- ▶ Strawberry cervix with punctate haemorrhagic lesions

Diagnosis should NOT be made only on the basis of clinical presentation

- ▶ The clinical symptoms may be identical to those seen in other sexually transmitted vaginal infections
- ▶ The classical 'strawberry cervix' with punctate hemorrhagic lesions is seen in approximately only 2% of patients
- ▶ The characteristic frothy discharge is seen in only 10% of women infected with *T. vaginalis*

If these features alone are used for the diagnosis of trichomoniasis

- ▶ 88% of infected women will not be diagnosed
- ▶ 29% of uninfected women will be mistakenly diagnosed

Accurate diagnosis is essential because

- ▶ Appropriate treatment can be given to the infected patients
- ▶ They facilitate the control of the spread of *T. vaginalis* infection

Hence combination of various microbiological tests should be employed for its diagnosis.

Suitable clinical specimens for laboratory diagnosis:

In females:

- ▶ High vaginal swabs from posterior fornix and lateral walls
- ▶ Vaginal secretions
- ▶ Endocervical smear/secretions

In males:

- ▶ Centrifuged deposit of urine
- ▶ Urethral discharge

- ▶ Urethral scrapings
- ▶ Semen
- ▶ Prostatic secretions

8.2 Collection of specimen

Collect the specimen preferably before the patient passes first urine in morning.

In females:

- With the help of a vaginal speculum visualize the posterior fornix.
- Collect the discharge from the posterior fornix with a sterile cotton wool swab soaked in normal saline.

In Males:

Provide the patient with a sterile bottle and ask him to collect first 20 ml of the urine passed first time in the day (early morning).

8.3. Transport of specimen

- Put the swab in 0.3 ml of saline if it is to be sent over a short distance not requiring more than 2 hours of transport.
- Put the vaginal swab in Amies' transport medium (Annexure-II) for transport to the laboratory if transport is expected to take more than 2 hours. The parasite remains alive in the transport medium for 24 hours at room temperature.

8.4 Microscopy

A. Wet Mount:

The wet mount should be examined within 15-20 minutes of collection as the parasite loses its distinctive motility due to variation in temperature.

Procedure:

- ▶ Suspend the secretions in 0.85% normal sterile saline
- ▶ Vigorously rotate swab in saline
- ▶ Press against the side of the tube to express as much fluid as possible
- ▶ Place one drop of the expressed fluid on a clean glass slide with a cover slip
- ▶ Examine the wet mount under the microscope

From urine prepare the wet mount by first centrifuging the urine at 1500 RPM for 10 minutes and then taking a drop of the deposit on a clean grease free microscope slide.

Observe the slide first under 10x magnification. Any field which shows the suspected organism is then seen under 40x magnification of light microscope.

Interpretation: The positive result is defined as the presence of one or more trichomonads with characteristic morphology and jerky motility.

The presence of *Trichomonas* is determined by the characteristic size $10 \times 7 \mu\text{m}$ of the organism, a pyriform shape with a lateral undulating membrane and the characteristic quivering / twitching motility.

Centrifuged samples do not show motility because the flagella are detached during centrifugation.

Specificity: 100%

Sensitivity: 35-80%.

Minimum concentration of 10^4 organisms per ml. of vaginal fluid appears to be necessary for identification of the protozoan by wet mount.

Wet mount examination may be employed routinely despite limited sensitivity because

- ▶ It is inexpensive
- ▶ It is quick
- ▶ Patients can be treated during the first visit

B) Staining:

- ▶ Prepare a smear of infected material on a clean grease free microscope slide.
- ▶ Allow it to dry in air, fix with methanol and stain.
- ▶ Giemsa, Papanicolaou, periodic acid-Schiff, acridine orange, fluorescein and immunoperoxidase stained smears can be used to detect *T.vaginalis* in asymptomatic women during routine examinations.
- ▶ Detection using the Papanicolaou test has reported sensitivity of 60% and specificity of 95–97%. Papanicolaou (Pap)(Annexure-I) staining may be the most practical approach for the detection of asymptomatic infections as a large number of asymptomatic women undergo routine cytological screening.
- ▶ Observe under oil immersion objective of light microscope after placing a drop of liquid paraffin on the smear.
- ▶ This method is less reliable than the wet mount for diagnosis.

Other supportive laboratory tests for diagnosis

8.5 Culture

- a. Culture is gold standard for the diagnosis of trichomoniasis.
- b. Perform culture in cases where vaginal wet mount preparation is negative and for

diagnosis of trichomonas infection in men because this is the most reliable and sensitive method of diagnosis. Although culture of *T. vaginalis* in men is preferred over wet-mount microscopy for diagnosis, the optimal specimen for culture is not clear. Studies indicate that sampling multiple urogenital sites substantially increases the detection of *T. vaginalis* in men. A practical approach is to combine a urethral swab specimen with first-voided urine sediment in a single culture.

- c. There are several media available for culture and studies have shown varying sensitivity and specificity.
- d. Store the prepared medium at 2-8°C.

I. Liquid /Semisolid media

Following media are used for culture of *T.vaginalis*:

- ▶ Diamond's Trichomonas Medium - Gold standard
 - ▶ Kupferberg Trichomonas Medium
 - ▶ Whittington Trichomonas Medium
 - ▶ TYM Medium
 - ▶ Hollander's modification of TY Medium
 - ▶ Dehydrated Trichomonas medium of Difco
 - ▶ TY1-S 33 medium
- } Other media

Refer Annexure-II for procedure on preparation of culture media.

Technique for culture

1. Inoculate the specimen in a liquid /semisolid/solid medium. For optimum growth fill long tube with liquid culture medium so as to provide anaerobic conditions at bottom and place the inoculum at the bottom of the tube.
2. Incubate the tube at 37°C.
3. Look for growth from the second day onwards. Most of the isolates become positive within 2-4 days.
4. Incubate the tube for 7 days before declaring it negative.

Interpretation

At intervals, macroscopically look for turbidity and microscopically examine the wet mount prepared from the material taken from the bottom of the tube for motile trichomonas. Giemsa stain may be performed to see the characteristic morphology.

The medium is equally suitable for the examination of urethral and vaginal swabs, and urine specimens.

Advantages of use of culture methods

- ▶ More sensitive than wet mount

II. InPouch TV culture system for diagnosis of Trichomoniasis

The InPouch TV is a double-pouched container made of soft, transparent plastic that allows one to perform a wet mount using the upper chamber and a culture using the lower chamber, in one self-contained system. The top pouch is inoculated with genital secretions suspected of having trichomonas and the specimen is pushed down into the bottom pouch, which serves as a container for the culture broth during the subsequent incubation.

Advantages:

- ▶ Simple procedure.
- ▶ Urine can be used instead of urethral specimen.
- ▶ Can be stored at room temperature up to one year.
- ▶ More sensitive than culture on other liquid/semisolid/solid media.

Procedure:

1. Collect the specimen from the posterior fornix of the vagina with a sterile swab.
2. Inoculate it in the top pouch of In Pouch for culture of *T. vaginalis* (InPouch TV is a double-pouched container made of soft plastic).
3. The specimen gets introduced into the bottom pouch immediately after collection.
4. Conduct microscopic examination of the bottom pouch when the specimen arrives in the laboratory (no examination is made of the top pouch because its contents had been immediately pushed into the bottom pouch).
5. Incubate the cultures at 35°C.
6. Examine for motile *T. vaginalis* at 24, 48 and 96 hours of incubation by using a 10X objective directly through the pouch.
7. Internal quality control for *T. vaginalis* InPouch culture is made by incubating one InPouch per batch as a sterility check on reagents and inoculating one pouch with a known culture of *T. vaginalis* to check the quality of the batch of InPouch.

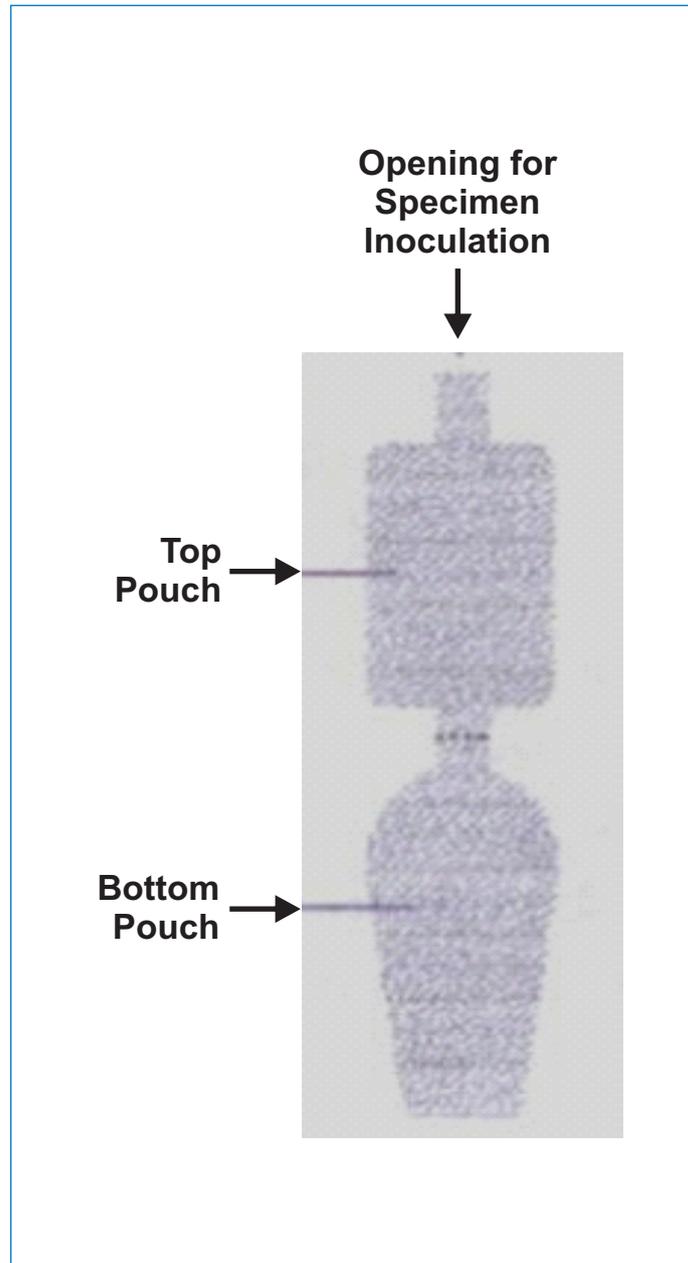


Figure 7 - In Pouch TV culture system for diagnosis of trichomoniasis

Nucleic acid amplification test (NAAT)

NAATs are preferred over conventional diagnostic methods for their superior sensitivity and specificity, shorter turnaround time, high throughput, and opportunity for automation, multiplexing, and quantification. Due to their high sensitivity, the NAATs also are effective in detecting organisms from asymptomatic infections or at early phase of infection and can be applied to self-collected and noninvasive specimens, such as vaginal swab and first-catch urine samples.

NAATs are available for detection of specific *T. vaginalis* DNA or RNA.

Rapid POC Tests

Commercial DNA-based microbial identification systems are available for detection of pathogens that cause vaginitis. Using synthetic oligonucleotide probes, these rapid tests simultaneously detect the presence of *Candida* species (yeast infection), *Gardnerella vaginalis* (bacterial vaginosis), and *Trichomonas vaginalis* from a single vaginal swab.

Sensitivity - 92%

Specificity - 98%

Rapid tests based on color immunochromatographic, capillary flow, "dipstick" technology are available, that detect antigens directly from vaginal swabs. The test is performed by inserting a vaginal swab into a test tube with special buffer, mixing the solution vigorously by hand, removing the swab, and then inserting a test strip. A visible blue test line is detected if *Trichomonas* antigen is present. No microscope is needed. Results are available within 10 minutes of administration.

SUMMARY

1. Observation of the parasite in the saline wet mount of the vaginal or urethral secretions confirms the diagnosis and can be done at the peripheral and intermediate level.
2. Microscopy confirms diagnosis in over 60% of symptomatic women.
3. In asymptomatic women and men, culture in liquid TV culture system, can be done at the intermediate and central level, especially in suspected patients. Culture can also be done in patients with clinically suspected infection, but with a negative wet mount examination.

9. HERPES SIMPLEX VIRUS (ANOGENITAL HERPES) TYPES -1 AND 2

9.1 Human (alpha) herpes virus 1 & 2 (HSV-1 & 2)

Herpes simplex virus type 2 causes genital herpes. It is a chronic infection with recurrent symptoms, and occurs as painful lesions of the genital organs. Genital herpes infections are mainly diagnosed by clinical examination.

9.2 Collection of samples

Materials required:

1. Fine tipped scalpel or scissors.
2. Clean, grease free microscope slides
3. Giemsa stain
4. Bright field microscope.

For microscopy:

1. Excise the top of a vesicle with a fine sterile scalpel or scissors.
2. Scrape the bottom of the ulcer with a scalpel or curette .
3. Prepare a smear on a clean, grease free microscope slide and allow it to dry in air.
4. Fix the smear with methanol.

For culture:

1. Open a large vesicle with a small (26) gauge needle.
2. Aspirate the fluid with a tuberculin syringe.
3. In women collect specimen from vagina with a sterile cotton wool swab.
4. Transfer the material into a vial containing 1ml of transport medium (Annexure-II)
5. Store at 4°C if the culture is to be performed within 48 hours.
6. Freeze at -70°C if the culture can only be done after 48 hours after collection.

9.3. Microscopy- Giemsa staining

1. Stain by Giemsa stain.
2. Observe under oil immersion of the microscope
3. Virus infected cells show ballooning of cytoplasm
4. Many cells fuse to give a multinucleated giant cell (MNGC).
5. Inclusion bodies are seen in the infected cells (Cowdry type A inclusions)
6. These inclusion bodies are present inside the nucleus (intranuclear), eosinophilic (red), granular in appearance and are of different sizes.

Immunoflourescent staining:

1. Method of choice for direct diagnosis of viral infection.
2. Kits are available for this technique. So only a main outline of the procedure is given.

3. Rub the swab with specimen on the slide provided with the kit.
4. Add fluorescein labelled monoclonal antibody to HSV 1& 2 to the slide.
5. Incubate at room temperature for 30 minutes.
6. Wash the slide with PBS (phosphate buffered saline), dry and mount the slide with a coverslip.
7. Examine the slide under a fluorescent microscope.

Reading:

Many yellow or yellow green shining (fluorescing) cells are observed.

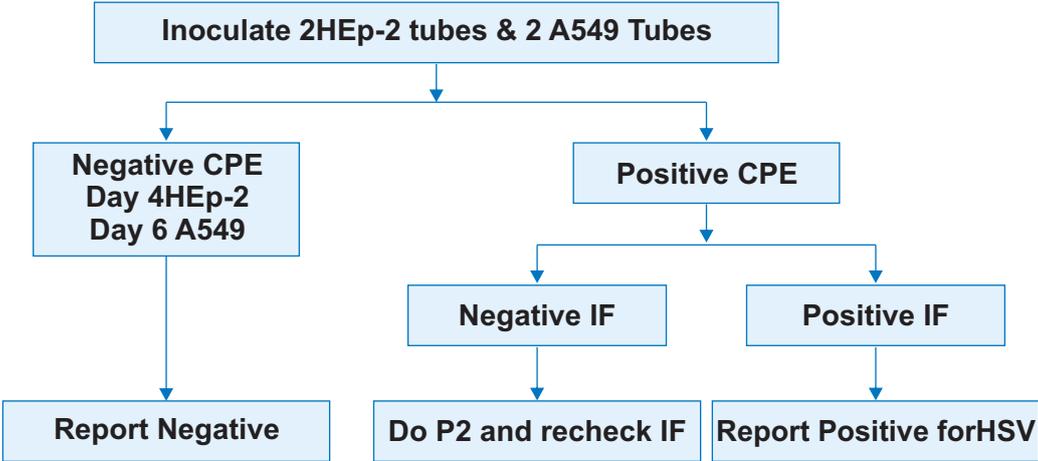
9.4 Antigen and Antibody detection

Different kinds of antigen and antibody testing techniques are available. Antigen detection is a good method for diagnosis when the patient presents with symptoms. Antigen detection can be carried out by ELISA, direct immunofluorescence and immunoperoxidase staining. IgM ELISAs antibody detection kits are useful to predict recent infection. ELISAs which detect type specific glycoprotein G (gG) are useful as they help in differentiating HSV-1 and HSV-2 infection. However, ELIASs have limited diagnostic value. Point of care (POC) rapid tests are commercially available for herpes antigen and antibody detection.

9.5 Viral culture

1. It is the most specific method to confirm infection due to HSV.
2. Cell lines used for the culture are human diploid fibroblasts (e.g MRC-5), VERO, HEp-2 and A549 (Human lung carcinoma) cell lines.
3. Take the monolayer cell line in a test tube which is flat on one side.
4. Remove the tissue culture medium and add 0.25 ml of the sample.
5. Keep the tubes in a horizontal position at 36°C for 20-30 minutes.
6. Add 2 ml of Culture Maintenance Medium to the tube and incubate at 36°C in an atmosphere of 5% CO₂.
7. Examine the tubes daily for next 7 days under a tissue culture microscope. Look for cytopathogenic effect in the form of :
 - ▶ rounding of cells
 - ▶ increase in the number of cells.
 - ▶ development of refractile appearance
 - ▶ Fusion of cells to give multinucleated giant cells.

Specimen genital swab/vesicle fluid/throat swab



9.6 Nucleic acid hybridization and amplification tests

Currently many laboratories employ NAATs to both detect and quantify HSV viruses in blood as well as body fluids. This is the most sensitive assay to diagnose HSV infection and is highly specific. These are available in many reference laboratories.

Viral DNA may be detected by hybridization techniques using radiolabelled or biotinylated probes.

These methods have largely been superseded by more sensitive and less laborious procedures which utilize amplification of the target HSV DNA by polymerase chain reaction (PCR).

Specificity of the amplification method is assured by either undertaking a second PCR with target-specific primers (nested PCR) or by HSV-specific probe hybridization of amplified products. In the case of possible genital herpes, PCR detects viral DNA for several days after lesions do not contain demonstrable infectious virus. This may mean that a laboratory switching to sensitive procedures based on nucleic acid amplification may have an increased number of positive results on lesion samples with possible clinical dilemmas regarding the relevance of positive results obtained after treatment.

With the recent advances in automation and kit developments for HSV detection and typing by PCR (eg, Real Art HSV1/2 kit from Artus-Biotech USA), it is likely that this methodology will become more widely used for routine diagnostic purposes.

SUMMARY

1. Microscopic examination of Giemsa stained smears should be attempted at the intermediate level laboratory. If it is not possible, then stained slides should be sent to the central laboratory.
2. Light microscopy is less reliable. So immunofluorescence technique should be used at the levels where fluorescent microscope, experienced staff and reagents are available.
3. The method of choice for the diagnosis of herpes simplex virus infection is culture. This should only be done in selected reference centres where facilities for tissue culture are available.
4. Nucleic acid amplification methods, drug resistance assays are available and are restricted to reference and research laboratories.

10. HUMAN PAPILLOMA VIRUS INFECTION

Human papilloma viruses cause genital warts (Condylomata acuminata). More than 100 types of HPV exist, more than 40 of which can infect the genital area. Most HPV infections are asymptomatic, unrecognized, or subclinical. Oncogenic or high-risk HPV types (e.g., HPV types 16 and 18), are the cause of cervical cancers. These HPV types are also associated with other anogenital cancers in men and women, including penile, vulvar, vaginal, and anal cancer, as well as a subset of oropharyngeal cancers. Nononcogenic, or low-risk HPV types (e.g., HPV types 6 and 11), are the cause of genital warts and recurrent respiratory papillomatosis. Asymptomatic genital HPV infection is common and usually self-limited; it is estimated that more than 50% of sexually active persons become infected at least once in their lifetime.

10.1 Non-oncogenic HPV infection

Genital warts are usually flat, papular, or pedunculated growths on the genital mucosa. Genital warts occur commonly at certain anatomic sites, including around the introitus in women, under the foreskin of the uncircumcised penis, and on the shaft of the circumcised penis. Genital warts can also occur at multiple sites in the anogenital epithelium or within the anogenital tract (e.g., cervix, vagina, urethra, perineum, perianal skin, and scrotum). Intra-anal warts are observed predominantly in persons who have had receptive anal intercourse, but they can also occur in men and women who do not have a history of anal sexual contact.

Diagnosis of genital warts is **usually clinical, made by visual inspection**. Genital warts can be confirmed by biopsy, which might be indicated if 1) the diagnosis is uncertain; 2) the lesions do not respond to standard therapy; 3) the disease worsens during therapy; 4) the lesion is atypical; 5) the patient has compromised immunity; or 6) the warts are pigmented, indurated, fixed, bleeding, or ulcerated. Genital warts are usually asymptomatic, but depending on the size and anatomic location, they might be painful or pruritic.

10.2 Oncogenic HPV infection

Oncogenic HPV causes cervical cancer. HPV is a sexually Transmitted infection and can remain undetected for years as it does not cause any symptoms. In order to detect cervical cancer very early, screening of women is indicated through cervical cytology.

10.3 HPV does not grow in cell culture. Hence culture is not available.

10.4 Detection of HPV DNA by nucleic acid hybridisation and amplification. This can be made available in central laboratory. Currently molecular methods of detection and genotyping of HPV has become the gold standard. There are three types of assays.

1. non-amplified hybridization assays such as Southern transfer blot, Dot-blot hybridization and in-situ hybridization.

2. signal amplified hybridization assays, such as hybrid-capture assays
3. target amplification assays

Many commercial assays are available based on the following principle for detection of HPV infection.

- ▶ Target amplification; genotyping; consensus PCR and line blot
- ▶ Target amplification; genotyping; PCR followed by line hybridization
- ▶ Target amplification; genotyping; SPF10 primers at L1 region, reverse hybridization
- ▶ Target amplification; detection; PCR and nucleic acid hybridization
- ▶ Target amplification of E1 for genotyping; PCR/DNA-array
- ▶ Signal amplification for detection; hybrid capture, semi-quantitative
- ▶ Signal amplification for detection; Invader technology

11. HEPATITIS B VIRUS INFECTION

Hepatitis B virus is a complex DNA virus having three different antigens. These are Hepatitis B surface antigen (HBsAg), Core antigen (HBcAg) and 'e' antigen (HBeAg). Presence of HBsAg in saliva, seminal fluid and vaginal secretions indicates its potential for spread during sexual activity.

Though hepatitis B infection covers many medical specialties, this manual is confined to its testing as part of STIs only.

Test	Method	Sample
HBsAg Antigen detection	Immunochromatography, lateral flow or other point of care test.	Serum
HBs Ag and Antibodies (Anti HBs)	ELISA, EIA, CLIA	Serum
Nucleic acid testing-detection	Real Time-PCR	Serum/ EDTA Blood
Viral nucleic acid quantification	Reverse Transcriptase-PCR	Serum/EDTA blood

Diagnostic testing

At primary level, immuno-chromatography or any other point of care test (follow procedures as per manufacturer technical instructions in the kit) can be used as a screening test for HBsAg. At intermediate laboratory level, ELISAs, or Chemiluminescence assays could be employed. Recently Enzyme Immuno Assay for quantitation of HBsAg has been approved and is expected to be performed at Intermediate level laboratories, which do not have molecular testing facilities. For further confirmation on activity of infection and detection of viral nucleic acid, refer samples/patient to central laboratory where the test is done.

12. HEPATITIS C INFECTION

Hepatitis C is a liver disease caused by the hepatitis C virus, which is a RNA virus. Though not primarily a sexually transmitted infection, it is also reported to be transmitted through sexual contact, exposure to infectious blood, blood products and organ transplants, injections given with contaminated syringes and needle-stick injuries in health-care settings, IV drug use, being born to a hepatitis C-infected mother. The virus has remarkable capacity to remain chronic and also be responsible for development of hepatocellular carcinoma.

12.1 Tests used in diagnosis of Hepatitis C infection

Test	Method	Sample
Antibody detection	Immuno chromatography	Serum
Antibody detection-IgG and IgM	ELISA, EIA, CLIA	Serum
Nucleic acid testing-detection	Real Time -PCR or TMA (Transcription Mediated Amplification)	Serum/ EDTA Blood
Viral nucleic acid quantification	Reverse Transcriptase-PCR	Serum/EDTA blood
Genotyping of HCV	Reverse Transcriptase-PCR	Serum/EDTA blood

12.2 Collection and transport of specimen

For serological assays collect blood in plain tubes, separate out serum and perform tests as per manufacturer. For Nucleic acid testing, follow instructions in manufacturer's kit.

12.3 Detection of antibodies to Hepatitis C virus

Anti-HCV antibody detection can be done by Immunochromatography or ELISA or Chemiluminescence immunoassays. Immunochromatographic tests can be done at primary level itself. All positives need confirmation of active infection by nucleic acid testing.

12.4 Viral nucleic acid detection

HCV RNA in blood can be detected and also quantified by polymerase chain reaction. It is a sensitive and specific test. Based on the kit used, follow instructions given by the central lab. Genotyping of virus also can be done to plan treatment regimen.

E. BIOSAFETY

Laboratory workers are at high risk for exposure to infectious agents. Infections can be acquired from exposure to contaminated blood, tissue and other samples during routine processing.

Laboratory practices that reduce the risk of infection are standard precautions, specific good laboratory practices, use of PPE, safety devices, proper decontamination, segregation and disposal of waste materials.

The main modes of transmission of an infectious agent of STI are:

1. Through cuts, bites, abrasions and injuries on the body surface causing a breach in the continuity of the skin that can happen while collecting samples as well as while processing them.
2. Through injury with skin piercing instruments like needles.
3. Through splashing of the contaminated material on the mucosa of the mouth, nose and eyes.

Some of the recommended practices to minimize laboratory acquired infections are as follows:

a. Laboratory area

1. An isolated spacious laboratory area should be limited to authorized personnel only.
2. A biohazard symbol should be prominently displayed at the entrance to the laboratory.
3. The laboratory surfaces like floor, walls etc. should be impervious to water and easily washable with chemicals and mild acids and alkalis.
4. The doors should be self-closing with a vision panel.
5. Sinks for hand washing should be easily accessible
6. Eyewash stations, showers should be available and easily accessible.
7. Eating, drinking etc. should be strictly prohibited in the laboratory area.
8. The windows should be covered with insect proof netting.
9. There should be enough illumination in the laboratory area.

b. Personal protective equipment

1. Laboratory coat/apron should always be donned while working.
2. Proper gloves should be used while handling contaminated material.
3. While handling infectious material, in addition to gloves, a mask and goggles should be worn as and when necessary.
4. Telephone calls should not be attended with gloved hands.
5. After handling or carrying out hazardous procedure, the hands must be immersed in a mild antiseptic solution and then washed with soap and water following all recommended steps of handwashing.
6. A complete shower immediately after going home and changing into fresh clothes is desirable.

c. Laboratory practices

1. Handling of specimens

- a. Wear gloves and gowns/aprons while handling samples, operating equipment
- b. Bandage open scratches
- c. Wash hands with soap and water immediately after sample collection. In absence of soap and water hand rubs or sanitisers may be used.
- d. Use needle locking syringes or safety devices while collecting samples
- e. Never recap or bend needles; discard them in puncture proof containers
- f. Carry tubes in a rack/basket always.
- g. Use plastic whenever possible instead of glass.
- h. No mouth pipetting should be done. Automatic pipetting devices should be used.
- i. Centrifuges should have covers so that there is no aerosol spray while using them.
- j. Screw capped bottles and tubes with stoppers should be used wherever applicable.

2. Disposal of contaminated materials

- a. All infected material should be rendered non-infective by autoclaving or chemical disinfection using 1% hypochlorite before discarding.
- b. Appropriate colour coded disposal bags and containers must be stationed in convenient locations of the laboratory. These include- Yellow bags for infectious waste, blue bags for infectious plastic waste and black bags for non-infectious materials like paper, card board etc. (Note- Follow local regulatory recommendations for colour codes.)
- c. All bags should be disposed of when 3/4 full and not left to brim over with waste. The mouths of the bags should be bunched up and secured before disposal.
- d. All contaminated/ infectious material (microbiological media with growth after disinfection, soiled gauze, swabs), should be disposed of in yellow bags and sent for incineration or any other appropriate method of final disposal.
- e. Needles, sharp instruments etc. must be discarded in a puncture proof container with sodium hypochlorite 1%. When the container is 2/3rd full, it should be closed and sent to the disposal agency for burial. If needle destroyers are present, then the needles may be destroyed after use and put in puncture resistant containers and disposed off in sharps pit.
- f. Soiled, disposable gloves must be cut / mutilated after use, to prevent reuse, and then disposed of in blue bags. Reusable gloves can be disinfected and autoclaved before reuse.

In summary-

Infectious, non-plastic waste → Yellow bags → Incineration

Infectious plastic waste → Blue bags/Red bags → Autoclave or Microwave/Disinfect → Shredder → Auctioned.

Infectious sharps → disinfect → white color sharps container → sharps pit.

Non infectious office waste → Black bags → Municipal collection → Sanitary landfill.

Liquid waste → disinfect → Drain/ sewer.

Note: Even if the waste disposal of a laboratory is outsourced to a biomedical waste treatment facility, the segregation of the waste is the responsibility of the laboratory personnel.

Local and state specific regulation to be followed wherever applicable for biomedical waste disposal.

3. Management of spills

- a. Every laboratory should have a spill kit in readiness. Contents of the spill kit include-cotton/gauze/absorbent paper + concentrated disinfectant + heavy duty gloves + waste lifting receptacle.
- b. Any contaminated material which spills should first be covered with adsorbent paper / gauze / cotton and then pour 1% Sodium hypochlorite. This should be allowed to remain for 20-30 minutes and then the paper should be removed and discarded, following this floor to be mopped with 1% sodium hypochlorite. The whole procedure should be done with gloved hands. The soiled waste should then be put in yellow bag and sent for incineration after securing it tightly.
- c. Broken pieces of glass, plastic etc. should be picked up with a pan and broom and thrown into a puncture-proof bin which should then be disposed of like sharps.

4. Management of sharps injury

In case of injury to a health care worker, steps to be followed:

- a. The site should be allowed to bleed freely.
- b. Never put the bleeding part to the mouth.
- c. Clean the injured site with soap and water/ spirit swab / dettol or savlon.
- d. The wound should then be covered with a waterproof dressing.
- e. The injury should then be reported to the laboratory in-charge/ supervisor.
- f. Appropriate PEP (Post Exposure Prophylaxis) should be followed as per NACO guidelines.
- g. In case of splashing on the mucous surface, the mucous surface should be washed with tap water and the accident reported to the laboratory in charge.
- h. All laboratory accidents should be recorded in accident register.

5. Training in safety

- a. A laboratory safety manual should be available in all laboratories.

- b. The staff should be trained in observing the safety precautions and the need for observing such safety precautions should be emphasized to them.
- c. All staff working with blood or serum should be immunized against Hepatitis B.
- d. Regular reorientation/ refresher programmes on safety measures and waste management should be conducted.

Preparation of Recommended Dilutions of Sodium Hypochlorite;

From 5% Stock Solution		
Required Volume of Working Solution (ml)	Amount of Stock Solution (ml)	Quantity of Water required (ml)
1% Working Solution of Sodium Hypochlorite		
1000	200	800
5000	1000	4000
0.1% Working Solution of Sodium Hypochlorite		
1000	20	980
5000	100	4900

F. WASHING AND STERILIZATION OF GLASSWARE

1. It is essential that all the glassware used for testing are cleaned well and are free of contaminating organisms, dust and extraneous interfering substances. Most of the containers (Petri dishes, tissue culture bottles, flasks, beakers etc.) and measuring devices (pipettes, measuring cylinders) used in a standard laboratory are of glass. So proper washing and sterilization of glassware is necessary to maintain high standards in an STI laboratory. If the glassware is not cleaned and sterilized properly then the results obtained after using this glassware will be erroneous. The glassware when new will be contaminated with spores and when used glassware is again utilized it may be contaminated with traces of previous culture. Hence the need for thorough cleaning and sterilization of glassware.

2. Preparation and cleaning of new glassware

- a. Put the glassware in a large plastic bucket containing 1% HCL (to neutralize the free alkali) and leave it overnight.
- b. Wash the glassware under running tap water.
- c. Wash the glassware with distilled water.
- d. Place it in an autoclave and sterilize at 121°C for 20 minutes at 15 pound pressure.

3. Cleaning of glassware used for bacterial culture

- a. Culture plates with growth should be autoclaved in a “waste” autoclave and then the growth disposed off in black bags. The petri dish and containers can then be washed and reused or disposed as general waste.
- b. Alternatively, autoclave at 121°C for 20 minutes at 15 lbs pressure the petri dishes, tubes etc. used for culture.
- c. If autoclaving is not possible, immerse in 3% Lysol or cresol or sodium hypochlorite 0.5-1%
- d. Boil the containers in a 5% solution of soap for 1 hour.
- e. Remove the glassware from soap solution and scrub with a brush.
- f. Rinse the scrubbed glassware in distilled water.
- g. Place on a rack to dry, pack in kraft/brown paper and sterilize in a hot air oven at 160°C for 1 hour.

4. Cleaning and sterilization of pipettes

- a. Take a container 15 inch high and 4 inch in diameter filled with 3% Lysol or 0.5%-1% sodium hypochlorite.
- b. Put the contaminated pipette in this container and leave for at least 30 min.
- c. Rinse in tap water.
- d. If pipettes are markedly contaminated, keep them in dichromate sulphuric acid solution overnight. Prepare the dichromate sulphuric acid solution by dissolving 63

gms of sodium dichromate in 35 ml of distilled water. Heat if necessary. Allow it to cool and add conc. H_2SO_4 to make the final volume to one litre.

N.B. Use thick rubber gloves and apron when preparing and using this solution.

- e. Wash with tap water with gloved hands.
- f. Draw in distilled water and discard.
- g. Draw in acetone and discard.
- h. Draw in air till the internal surface is dry.
- i. Keep the pipettes in copper cylinder with lid.
- j. Sterilize in hot air oven at $160^{\circ}C$ for 60 min.

5. Cleaning of glassware for tissue culture

Tissue cultures are sensitive to alkalinity of glass. Hence thorough cleaning of glassware before use is essential.

A) New glassware:

1. Keep the glassware for 1 hour in a large plastic bucket having 25% 1 N H_2SO_4 .
2. Rinse 6 times with tap water
3. Boil in soap solution for 15 minutes.
4. Wash 6 times with tap water.
5. Keep the glassware for 1 hour in a large plastic bucket in distilled water with 0.05% 1N HCL.
6. Wash the glassware 3 times with distilled water.
7. Place the glassware on a rack to dry and wrap in aluminum foil after drying.
8. Sterilize by dry heat at $160^{\circ}C$ for 1 hour.

B) Old glassware:

1. Clean the glassware with soap solution.
2. Wash 6 times with tap water.
3. Place the glassware for 1 hour in a large plastic bucket in distilled water with 0.05% 1N HCL (This neutralizes the alkalinity resulting from the use of soap)
4. Wash 3 times with distilled water.
5. Dry the glassware, wrap in aluminum foil and sterilize by dry heat at $160^{\circ}C$ for 1 hour.

C) Glassware containing tissue culture:

1. Autoclave the container with rubber stoppers at $121^{\circ}C$ for 20 min with 15lbs.pressure.
2. Scrub the container with a brush and wash in tap water. This removes the cellular material.
3. Place in a mild soap solution and boil for 15 minutes.
4. Remove the soap solution and wash 6 times in tap water.

5. Keep the glassware for 1 hour in a large plastic bucket in distilled water with 0.05% 1N HCL.
6. Wash 3 times with distilled water.
7. Dry the glassware, wrap in aluminum foil and sterilize by dry heat at 160°C for 3 hrs.

D) Rubber Stoppers:

1. Wash the autoclaved rubber stoppers with hot tap water.
2. Keep the rubber stoppers in 20% NaHCO₃ solution and boil for 20 min.
3. Wash the stopper with hot tap water.
4. Keep the rubber stoppers for 1 hour in 0.05% in HCL.
5. Wash in hot tap water.
6. Place in a tin or aluminum container after drying and autoclave at 121°C for 20 min under 15 lbs pressure.

G. MAINTENANCE OF EQUIPMENT

Maintenance of equipment in good working condition is absolutely essential for the smooth functioning of laboratory. Preventive maintenance and calibration of equipment are generally done by authorized service personnel. Routine maintenance and function checks are done by the laboratory technicians who work with the equipment, under the supervision of technical officer or laboratory in-charge.

All the equipment should have adequate documentation on its maintenance, repairs, downtime and calibration. Equipment history card should be maintained for every equipment and it should have:

- ▶ Name of the equipment
- ▶ Unique ID, model and the serial no
- ▶ Manufacturer name and address
- ▶ Service person's name, address and contact no
- ▶ Date of installation
- ▶ Calibration frequency, calibration certificates
- ▶ Repairs and service details
- ▶ Every equipment should have work instructions for its operation and troubleshooting guide available at the workplace.

Calibration of equipment:

All the equipment except centrifuges and micropipettes should be calibrated annually. The laboratory should have an annual calibration plan which helps in timely calibration of equipment. Centrifuges and micropipettes should be calibrated every six months . All calibrations should be done by the manufacturer or an accredited calibrating agency/laboratory. Records should be maintained.

Some of the common equipment and their maintenance protocol is given below:

1. **Microscopes (Light, Dark field and Fluorescent):** Every time the microscope is used, the lenses should be cleaned with a lens paper/soft gauze soaked in xylene. Alcohol should not be used to clean the optical system because it dissolves the cement material and thereby detaches the lenses. The whole microscope can be cleaned with a clean gauze to get rid of the dust particles, every time it is used. Special care should be taken to avoid damage to the light source of dark field and fluorescent microscope. When the microscope is not in use, use of a dust cover is desirable. Optical system should be cleaned by a trained/ authorized person atleast once in six months AMC / CMC is desirable.
2. **Centrifuge:** It should always be placed in a horizontal position, the safety cover should be closed when in use. It should be cleaned regularly with gauze. Calibration of the centrifuge should be done every six months for the speed and timer with a calibrated tachometer.

3. **Refrigerators (4 to 8°C) and freezers (-20 to -80°C):** Calibrated thermometers should be installed and the temperature recorded daily. They should be defrosted at regular intervals, if indicated.
4. **Ovens, Incubators and Heat Blocks:** These should be checked daily and temperature recorded daily. Marked fluctuations should be reported to the maintenance technician. Sterility check for ovens should be carried out every time the equipment is used.
5. **Water baths:** The temperature should be checked daily and recorded. The water level should be maintained at optimum levels and checked daily.
6. **Thermometers (0-100°C):** The thermometer should be checked routinely for precision against the thermometers provided by the Indian Standard Institute (ISI).
7. **Autoclaves:** They should be checked daily, the temperature achieved should be recorded daily and sterility check by chemical indicator should be done every time and biological indicator every week. Pressure and temperature gauges should be calibrated annually.
8. **Rotators:** The VDRL rotator should be checked every time it is used by placing the finger vertically over it and counting the RPM. The RPM should be adjusted to the correct speed.
9. **CO₂ incubator or candle jar (desiccator):** In CO₂ incubator, check for temperature and CO₂ conc. regularly. Glass candle jars should be checked for any cracks. Also the grease coating on the lid should be checked because enough grease on the lid margins is essential for airtight closure of the lid.
10. **Volumetric equipment and automatic pipetting devices:** These should be checked every month and calibrated every six months for the required volume delivery. The calibration may be done in-house or outsourced to an external agency that is traceable to an accreditation body.
11. **Analytical balance:** It should be checked daily or weekly, depending on the frequency of use and should be calibrated annually with weights obtained from Indian Standard Institute.
12. **pH meter:** It should be calibrated daily and used subsequently after a series of known buffers are tested and the record of the result should be maintained.
13. **Biological safety cabinet:** If being used, should be decontaminated daily by exposure to UV light. Sterility check should be done weekly by placing exposed plates inside the cabinet. Integrity of HEPA filter, airflow velocity should be checked annually.
14. **ELISA reader and washer:** Should be checked every time it is used by first recording the optical density of a blank control. If the optical density is beyond permissible limits, the instrument should immediately be checked by an experienced technician/service engineer. Annual calibration of ELISA reader includes checking of filters and alignment of optical path and filters.

H. QUALITY ASSURANCE AND QUALITY CONTROL

1. Quality Assurance

Quality assurance indicates an overall plan of activities that are undertaken to demonstrate that quality is ensured in the entire process flow so that the results are accurate and reproducible.

This plan consists of:

1. Organization and management
2. Availability of validated standard operating procedures
3. Document control, proper recording of data
4. Training and competency assessment of staff
5. Equipment maintenance and calibration
6. Monitoring of purchase and inventory
7. Environment and personal safety
8. Internal quality control procedures
9. Participation in external quality assurance programs.
10. Procedures to identify non-conformities, complaints and take corrective action
11. Risk assessments and preventive actions
12. Quality improvement through indicators

The QA program ensures that correct procedures are followed throughout pre-analytical, analytical and post-analytical phases of laboratory operation.

2. Quality Control (QC) consists of routine, planned activities that are undertaken to ensure accuracy and precision of all test results. This is achieved through internal quality control procedures, external quality assurance programs and inter-laboratory comparisons.

3. Quality Assurance activities can be broadly brought under three categories

A. Structure:

- 1. Organisation and leadership:** The laboratory should have a proper organization structure with assigned responsibilities, leadership, vision, and resources (finance, human and policies) to fulfill the targets.
- 2. Personnel:** Proper defined and documented job qualifications, job description, orientation and training of staff, competency evaluations, provision of continuing education opportunities should be in place. All staff should have a structured induction training and annual competency evaluations by processing of split samples.
- 3. Environment and accommodation:** Adequate space should be available with segregation of activities, appropriate temperature, humidity, fire, chemical and bio-safety programs and communication channels. In order to ensure safety, there should be a designated laboratory safety officer whose responsibility is to train the staff and oversee safety practices and monitor biomedical waste disposal.

B. Process:

- 1. Purchase and inventory:** Identification of critical materials and supplies, supplier qualification, periodical evaluations, inventory management, checking of new lots for their required performance.
- 2. Equipment:** Selection and acquisition of suitable equipment, correct installation, performance checks, periodical calibrations, maintenance, protection from contamination.
- 3. Analytical procedures:** Choice of validated procedures, availability of SOPs, review of SOPs, process validation, quality controls, (see below for policy on quality control) change control.

C. Outcome:

Identification of nonconforming activities, root cause analysis, corrective and preventive actions, risk assessments, complaint mechanisms, audits and monitoring of quality indicators and management review.

All the above activities should be detailed in policy and procedure manuals such as Quality System manual, safety manual and technical SOPs. These manuals are specific to the laboratory and need to be written in line with local conditions.

Policy on Internal Quality Control:

1. All the newly purchased/prepared reagents or kits should be put to routine testing only after they have been tested with quality control materials or retained samples and approved.
2. All the tests that are done should have positive and negative controls run with each batch of testing done. In case of serological assays, the controls should also include low-positive /grey area control to make sure that the laboratory is able to get reproducible results at all levels.
3. Assays that are quantitative, should have their standard deviations calculated, %cv derived and monthly LJ charts should be monitored to check trends/bias.

External quality assurance for STI/RTI laboratory network:

1. It is recommended that the apex reference laboratory is given the responsibility of planning and running an EQA program for the regional reference laboratories and regional reference laboratories for the state reference laboratories.
2. A quality assurance program developed by the apex and regional reference laboratory should have the following components: Sending out samples for testing, analyzing the reports based on the methodology, equipment used and giving feedback on results obtained. Efforts would be taken to get this EQA program accredited over a period of time.
3. In addition, surveillance of test reports could be done by rechecking all positive samples and 5% of negative samples at all levels of laboratories in the following format:

Laboratory	Quality assurance at	Frequency	Method
Primary level	SRC	Biannually	Retesting of all positives (Slides and serum samples) and 5% of negatives
DSRC	SRC	Biannually	Retesting of all positives (Slides and serum samples) and 5% of negatives
SRC	RSTRRL	Biannually	EQA program
RSTRRL	Apex laboratory	Biannually	EQA program

I. REGISTRATION AND RECORD KEEPING

Registration and record keeping are an integral part of the laboratory services. The basic data regarding the names, address, sex, husband's/ father's name and age are registered at the point of entry of the patient.

The STI counselors maintain a record of the individual patient in the STI card allotted after the registration is done, which not only provides the basic data but also the medical history, presenting signs and symptoms, findings on physical examination, laboratory investigation done and its result, diagnosis, treatment and follow-up visits. This will allow physicians to provide quality medical care, and will also assist in the identification of repeat offenders.

Confidentiality with regard to the results of laboratory test results is of utmost importance. Only authorized persons can enter and access the STI card record of the patients so as to maintain the confidentiality and a healthy patient doctor relationship. Not only is breach of confidence punishable by law, it is also very damaging to health worker-patient relationship.

Laboratory registration and record keeping serves following purposes:

1. To ensure that the right report reaches the right person without any confusion if two or more patients of the same name attended the STI clinic at a point of time.
2. To record the work load in the laboratory, keeping track of the number of different tests performed.
3. To prepare the monthly report of the laboratory.
4. To know the requirements of the reagents, kits etc. on a monthly basis so as to maintain adequate stock for continuation of work without any interruption.

The following steps should be taken in recording and registration:

1. Specimen tubes, slides and containers should be labeled with the name of the patient or with the serial number on the form, if patient identification is by number rather than the name.
2. All incoming request should be entered in register, preferably using a different register for each kind of test or procedure.
3. Test result should be entered on the request form, as well as in the recording registers (rough and reporting).
4. All the laboratory reports should be signed by an authorized person.
5. The test result should be sent back to the requesting physician. A record should be kept for outgoing reports. This may be done in the same book where the test and its result are recorded.
6. Monthly tallies can be made of the number of different tests or procedures performed, and of the results.

7. Any quality control tests done should also be recorded.
8. Records of tests and results should be kept confidential, especially if they are recorded under the name of the individual patients, rather than under numbers. It is clear that records kept under numbers provide better guarantees for confidentiality than those kept under name. Record books should be kept in locked cupboards.
9. Computerized record should be protected against unauthorized access to the file and should also be maintained in duplicate, so that if, by mistake, the information is erased, a copy is still available.
10. Any problem faced during the test should also be recorded with the action taken.
11. Also, the records of temperature wherever applicable must be maintained.
12. Records of the accidents occurred, if any, and corrective action taken should also be maintained for future reference.

ANNEXURE - I

MICROSCOPY

1) Preparation of smear

- ▶ Take a clean grease free microscope slide and wipe it with gauze piece.
- ▶ Pass it through the flame twice or thrice and again wipe it clean. This removes the excess grease on the slide.
- ▶ With a glass marking pencil, draw two vertical lines, 2.5 cms apart in the central part of the slide.
- ▶ Roll the cotton wool swab with the specimen on to this marked area.
- ▶ Spread it to an area of 2 cms x 1 cms. Smears should neither be too thick nor too thin. It should be of such thickness that a bold newspaper print, when viewed from below, should be readable. The smears should not extend to the edges of the slide.
- ▶ For the culture smear; first take a drop of normal saline on the slide. Then with the help of a heat-sterilized platinum/nichrome loop, just touch the suspect colony. Prepare a uniform emulsion of the organism by mixing it with the help of saline on the slide. The loop should be heated red hot in a flame and allowed to cool before picking up the colony. Similarly after preparing the smear, the loop should be sterilized by heating it red hot.
- ▶ Allow the smear to dry in the air before heat fixing.
- ▶ Label the smear on the right or left hand corner of the slide.

Note: The cleanliness of the slide can be judged by placing a drop of water over it and spreading this drop. If the slide is clean and free of grease, this drop spreads into a thin even film; but if the slide is not properly cleaned, the water collects on the slide in the form of a fine droplet and a film cannot be made.

2. Fixing of smear

a. Heat fixing:

- a) Hold the slide film upwards
- b) Pass it over the flame of a Bunsen burner or a spirit lamp twice or thrice in quick succession.
- c) Feel the temperature on the back of the hand (dorsum of hand). When the slide is just hot enough to be tolerated, fixing is complete. Too much heating chars the smear and alters the morphology of the organisms. Less heating fails to fix the smear and it may be washed away during the staining procedure. Fixing kills the organism, fixes it to the slide, prevents autolytic changes, makes the organism permeable to the dye and harmless to the person handling the smear. After heat fixation, the film is stained.

b. Methyl alcohol fixing (Methanol fixing):

- a) Fill a Coplin jar with methyl alcohol.

- b) Take the slide with the film to be stained and allow the film to dry in air.
- c) Place this slide in methyl alcohol for 3 minutes.
- d) Remove after 3 minutes from the Coplin jar, allow it to dry in air and stain the film.
- e) Fixing with methanol preserves the morphology of cells especially when leucocytes are to be reported.

3. Stains and staining procedures

a. Gram's staining

Reagents:

- 1) Crystal violet

Solution A: Crystal violet powder	20 gm
Ethyl alcohol	200 ml
Solution B: Ammonium Oxalate	8 gm
Distilled water	800 ml
Mix solution A and B then filter.	
- 2) Gram's iodine

Iodine crystals	3 gm
Potassium iodide	6 gm
Add 900 ml distilled water	
- 3) Acetone alcohol

Acetone 100 %	100 ml
Absolute ethyl alcohol	100 ml

Acetone and alcohol may be used alone or in various ratios (1:4, 1:2, 1:1). Higher the acetone content, more rapid is the decolourisation.

- 4) Safranin

Stock solution: Safranin	10 gm
Ethyl alcohol	200 ml
Working solution: Stock solution	100 ml
Distilled water	900 ml

Alternatively,

Safranin powder:	10 gm
D/W:	1000ml

Make a paste of safranin powder in 50 ml D/W. Then make volume upto 1000 ml with D/W. Filter and use.

In addition to the In-house preparation, ready to use commercially available stains may be used.

Procedure:

- 1) Primary staining- Cover the slide with Crystal/Gentian violet solution and allow to act for upto 1 min.
- 2) Pour off the stain, and wash with water. Cover the slide with Gram's iodine and allow it to act for about 1 min.
- 3) Decolourisation- Wash off the iodine and wash with water. Add acetone alcohol. Tilt the slide from side to side till violet colour ceases to come off the slide. This can be confirmed by holding the slide against a white background. Do not decolourise for more than 10-15 secs.
- 4) Absolute alcohol (100% ethanol) can be used instead of acetone alcohol for decolourisation.
- 5) Wash the slide with water.
- 6) Counter staining- Pour safranin on the slide and allow to act for 30 secs.
- 7) Wash with water; blot the slide dry between two blotting papers.
- 8) Put a drop of immersion oil on the smear and observe under oil immersion lens.

For gonococcus Sandiford's counterstain is useful, particularly when the organisms are few in number. Neutral red can also be used as a counterstain for gonococcus.

Sandiford's counterstain

Malachite green	0.05 gm
Pyronine	0.15 gm
Distilled water to	100 ml

The stain keeps for about a month.

Stain the slide in the usual manner. Instead of safranin, apply Sandiford's counterstain for 2 minutes, flood off with water (do not wash) and blot.

Reading:

Cells and Nuclei	Bluish green
Gram positive organism	Purple black
Gonococci (and other Gram negative organisms)	Red to pink

b. Giemsa staining**Reagents:**

- 1) Giemsa powder 1.0 gm

Dissolve the Giemsa powder in 66 ml of Glycerol. Heat the mixture at 56 deg C for 90-120 mins. Cool it to room temperature. Add 66 ml of Methanol and mix thoroughly and allow to stand for 7 days at room temperature (or 37 deg C). Filter. It is now ready for use after making a working solution with buffer.

- 2) Buffer : Dilute: 1/15 M phosphate buffer, pH 7.0 prepared as follows:
- | | | |
|----|-----------------------------------|---------|
| a) | Na ₂ HPO ₄ | 9.5 gm |
| | Distilled water | 1000 ml |
| b) | Na H ₂ PO ₄ | 9.2 gm |
| | Distilled water | 1000 ml |

Mix 72 ml of solution (a) with 28 ml of solution (b)

Procedure:

Rapid Method:

- 1) Take the methanol fixed smear and stain with 1 part stain and 9 part buffer solution for 30 minutes.
- 2) Wash the slide with buffer for about 30 second.
- 3) Blot the slide and allow to dry in air.

Slow Method:

- 1) Take the methanol fixed smear on slide and place it in a Petri dish with one end on a glass rod, film downwards.
- 2) Mix 1 ml of the stain with 20 ml of diluent buffer and pour into the Petri dish so that there is sufficient stain between the film and the bottom of the Petri dish.
- 3) Leave the slide in the stain overnight (16-24 hours).
- 4) Wash the slide with buffer, allow it to dry in the air, mount and observe under oil immersion lens of the microscope.

c. Papanicolaou Staining

Reagent:

- | | |
|----------------------------|---|
| Fixative: | Equal parts of 95 % alcohol and ether. |
| 50 % Alcohol : | Diluted 50 ml of 95 % alcohol to 95 ml with distilled water. |
| 70 % Alcohol : | Diluted 70 ml of 95 % alcohol to 95 ml with distilled water. |
| 80 % Alcohol : | Diluted 80 ml of 95 % alcohol to 95 ml with distilled water. |
| 100 % Alcohol : | Mix Absolute alcohol with pulverized and previously dried Copper sulphate (CuSO ₄). The Copper Sulphate settles to the bottom of the bottle and 100% alcohol remains above. |
| Harris Haematoxylin: | Recommended here as a nuclear stain. |
| Haematoxylin: | crystal 1 gm |
| 95 % alcohol | 10 ml |
| Ammonium or potassium alum | 20 gm |
| Distilled water | 200 ml |

Dissolve the Haematoxylin in the alcohol. Dissolve the alum in the distilled water using heat if necessary and then add the Haematoxylin solution. Bring the solution to boil and add 0.5 gms of mercuric oxide. The solution becomes dark purple. Remove the stain containing vessel from the flame and cool it immediately by placing in a bath of running cold water. After cooling the solution is ready for use. Addition of 3 ml of glacial acetic acid to 95 ml of the solution at the time of use increases its nuclear staining properties. 0.5 % Aqueous Hydrochloric Acid Solution Add 0.5 ml hydrochloric acid to distilled water so that the final volume is 100 ml.

Orange G:

Dissolve 0.5 gms of Orange G in 100 ml of 95 % of alcohol and add 0.015 gms of phosphotungstic acid (if Orange G 6 is used; if Orange G 8 is used, add 0.010 gms of the acid).

Stain EA 36:

0.5 % alcoholic light Green SF Yellow	45 ml
0.5 % alcoholic Bismarck brown	10 ml
0.5% alcoholic eosin yellow	45 ml
Phosphotungstic acid	0.2 gms
Saturated aqueous solution of lithium carbonate	1 drop

Stain EA 25:

0.5 % alcoholic light Green SF Yellow	44 ml
0.5 % alcoholic Bismarck brown	12 ml
0.5% alcoholic eosin yellow	44 ml
Phosphotungstic acid	0.17 gms
Saturated aqueous solution of lithium carbonate	1 drop
Mounting medium :	Canada balsam

Procedure:

Make 0.5 % alcoholic solution first. Because the solubility of the dyes in 95 % alcohol is low, heat the solution at the time of preparation. Keep the solution in stock without filtering. Filter stains EA 36 and 25 to remove undissolved stain particles.

- 1) Fix smears immediately (before drying) in equal parts of alcohol and ether for 5 to 10 minutes
- 2) Carry out the staining process in Coplin jars or staining dishes.
- 3) After fixation, transfer the slides immediately, without drying, directly from the alcohol –ether mixture to 80 % alcohol.
- 4) Dip the slide 4 times in the coplin jar with 80 % alcohol.
- 5) Dip the slide 4 times in the coplin jar with 70 % alcohol.
- 6) Dip the slide 4 times in the coplin jar with 50 % alcohol.
- 7) Dip the slide 4 times in the coplin jar with distilled water.

- 8) Stain the slide in Harris haematoxylin for 6 minutes.
 - 9) Rinse the slide in distilled water.
 - 10) Dip the slide 6 times in 0.5% hydrochloric acid solution.
 - 11) Wash the slide in running distilled water for 6 minutes.
 - 12) Rinse the slide in 50% alcohol.
 - 13) Rinse the slide in 70% alcohol.
 - 14) Rinse the slide in 80% alcohol.
 - 15) Rinse the slide in 95% alcohol.
 - 16) Stain the slide in Orange G solution for 90 seconds.
 - 17) Rinse the slide 5-10 times in a jar of 95% alcohol,
 - 18) Rinse the slide 5-10 times in another jar of 95% alcohol to remove excess stain.
 - 19) Stain the slide in EA 36 or EA 25 for two minutes.
 - 20) Rinse the slide 5-10 times in a jar of 95% alcohol,
 - 21) Rinse the slide 5-10 times in a second jar of 95% alcohol.
 - 22) Rinse the slide 5-10 times in a Third jar having 95 % alcohol.
 - 23) Rinse the slide in absolute alcohol.
 - 24) Rinse the slide in alcohol-xylol.
 - 25) Rinse the slide in xylol.
 - 26) Mount the slide in Canada Balsam.
 - 27) Nuclei of cells stain deep blue or purple.
 - 28) Cytoplasm (Basophilic material) stain light blue or blue green.
- Keratinised cells show various shades of Red (Acidophilic cells-Red to Orange).

Rapid-Diff Staining.

Reagents:

- | | | |
|---------------|-------------------|-----------------------------|
| 1. Solution A | Fixing Solutions: | Thiazine Dye in Methanol |
| 2. Solution B | Acid Dye: | Eosin Y in Phosphate Buffer |
| 3. Solution C | Basic Dye: | Thiazine Dye Mixture |

Procedure:

1. Prepare the smears on grease free slide, and air dry.
2. Place the smear in RapiDiff fixative solution A (RapiDiff fixative) for 15 seconds (6 dips).
3. Transfer, without rinsing or drying, to solution B (Eosin Y) and stain slowly agitating the slide in the solution or immersing and withdrawing the slide several times during for 15 seconds period. Drain excess stain onto absorbent paper.
4. Transfer slide to solution C (Thiazine Dye Mixture) and stain as slowly agitating the slide in the solution or immersing and withdrawing the slide several times during for 15 seconds period.
5. Finally rinse the slide in Phosphate buffer pH 6.8.
6. Air dry the stained slide and examine by light microscopy under oil immersion (1000 x Magnification).

ANNEXURE - II

Media and reagents

For *Neisseria gonorrhoeae*

1) AMIES TRANSPORT MEDIUM

Ingredients:

Sodium thioglycollate (mercaptoacetate)	1.0 gm
Sodium chloride (NaCl)	3.0 gm
Potassium chloride (KCl)	0.2 gm
Calcium chloride (CaCl ₂)	0.1 gm
Magnesium chloride (MgCl ₂ 6H ₂ O)	0.1 gm
Disodium hydrogen phosphate (Na ₂ HPO ₄)	1.15 gm
Potassium dihydrogen phosphate (KH ₂ PO ₄)	0.2 gm
Charcoal (finely powdered, pharmaceutical)	10 gm
Agar	4.0 gm
Distilled water	1000 ml

Procedure:

- 1) Suspend the chemical salts, agar and charcoal in 1000 ml of distilled water.
- 2) Boil to dissolve the agar completely.
- 3) Check the pH (7.2).
- 4) Distribute into bijou bottles filling them completely. Keep stirring during distribution (This keeps the charcoal uniformly suspended).
- 5) Sterilize by autoclaving at 121°C for 15 minutes.
- 6) Cool immediately in cold water (This keeps the charcoal uniformly suspended).
- 7) During cooling, keep inverting the bottles frequently (This again helps in uniform suspension of charcoal).

2) STUART'S TRANSPORT MEDIUM

Ingredients:

Sodium thioglycollate	1.0 gm.
Sodium glycerophosphate	10 gm.
Calcium Chloride	0.1 gm.
Methylene blue	0.002 gm.
Agar	3.0-5.0 gm.
Distilled water	1000 ml.

Procedure:

- 1) Mix the contents in distilled water and boil.
- 2) Distribute into small screw capped bottles after adjusting the pH to 7.3 to 7.4.
- 3) Autoclave at 121° C for 15 min. and immediately tighten the cap.
- 4) The medium should be colourless when cool.
- 5) Store in refrigerator till further use.
- 6) If the colour changes to blue on storage, it indicates that medium is aerated and unfit for use.
- 7) Loosen the screw cap and heat the medium to remove the trapped air.

3) CHOCOLATE AGAR**Ingredients:**

Columbia agar base	44 gm.
Distilled water	1000 ml.
Horse blood/Sheep blood	90 ml/100 ml.

- 1) Dissolve 44 gm of Columbia agar base to 1 litre of distilled water in a flask. Heat in a steam sterilizer to dissolve it completely.
- 2) Adjust the pH to 7.5 – 7.6.
- 3) Sterilize by autoclaving at 121° C for 15 minutes.
- 4) Cool to 70° C in a water bath.
- 5) Aseptically add 90 ml of defibrinated horse blood or 100 ml of sheep blood to it and leave at 70° C for 30 minutes.
- 6) Mix the blood and agar by gentle agitation from time to time till blood becomes chocolate brown in colour.
- 7) Transfer to a 50° C waterbath to cool before pouring.
- 8) Pour as slopes or plates in sterile tubes or sterile Petri dishes.

4) SAPONIN LYSSED BLOOD AGAR WITH VCNT INHIBITORS

A selective medium for the growth of *N. gonorrhoeae*.

Ingredients:

- 1) GC agar base 36 gm
- 2) Saponin lysed Horse blood/Sheep blood 90 ml (9% final conc.)
- 3) Distilled water 1000 ml.
- 4) VCNT inhibitor 10ml
 - i) Vancomycin 3.0 mg or lincomycin 1 mg/litre
 - ii) Colistin 300,000 units/7.5 mg/litre
 - iii) Nystatin 12.5 IU/ml
 - iv) Trimethoprim 2mg./litre.

Procedure:

- 1) Mix 36 gms GC agar single strength in 1 litre of distilled water by boiling.
- 2) Sterilize by autoclaving at 121° C for 15 minutes at 15 lbs. pressure.
- 3) Cool to a temperature of 50-55° C in a waterbath.
- 4) Aseptically add saponin lysed horse or sheep blood and VCNT inhibitor and mix well.
- 5) Pour 20 ml of medium in a sterile Petri dish of 90 mm diameter under strict aseptic precautions.
- 6) Allow the agar to set and store the plates inverted in refrigerator.

SAPONIN LYSED SHEEP BLOOD

Add 6.75 ml of 10% saponin solution to 90 ml defibrinated sheep blood and mix gently. Leave in an incubator at 37°C for approximately 20 minutes, gently mixing occasionally until the blood is lysed.

10% SAPONIN

10% saponin is prepared by dissolving 5 gm saponin in 50 ml distilled water. Sterilize by autoclaving at 121° C for 15 minutes.

5) BUFFERED BALANCED SALT INDICATOR SOLUTION (BSS)**Ingredients:**

Dipotassium hydrogen phosphate	0.4 gms
Potassium dihydrogen phosphate	0.1 gm
Potassium chloride	8.0 gms
Phenol Red indicator	0.1 gms
Distilled water	1000 ml

Procedure:

- 1) Mix the salts and indicator in distilled water.
- 2) Adjust the pH to 7.1 to 7.2.
- 3) Autoclave/Sterilize by Seitz filter.
- 4) Store in the refrigerator at 4°C.

6) PHOSPHATE BUFFERED SALINE:**Ingredients:**

Sodium chloride	8.0 gm
Potassium chloride	0.2 gm
Disodium hydrogen phosphate	1.44 gm
Potassium dihydrogen phosphate	0.24 gm
Distilled water	1000 ml

Mix the salts in distilled water to give a final pH 7.3.

Chlamydia trachomatis

1) SUCROSE PHOSPHATE TRANSPORT MEDIUM

(Phosphate buffered sucrose medium):

It is made up of a buffered sucrose base and supplements.

1) Base:

Dipotassium hydrogen phosphate	2.1 gms
Potassium dihydrogen phosphate	1.1 gm
Sucrose	68.5 gm
Distilled water	1000 ml

The pH achieved is 7.2.

Mix the salts in distilled water and adjust the pH to 7.2.

Sterilize by filtration.

Dispense 90 ml in one aliquot aseptically.

To one aliquot of 90 ml add the following supplements aseptically to prepare the Complete medium.

2) Supplements:

Foetal Calf serum	10 ml
Gentamicin	10 mg
Vancomycin	10 mg
Amphotericin B	0.5 mg

2) STANDARD GROWTH MEDIUM (Chlamydia cell growth medium)

Ingredients:

Eagle minimum essential medium	14.49 gm
Sodium bicarbonate (7.5 %)	7.5 ml
L-Glutamine (200 mmol/ ml)	10 ml
Gentamicin (50 mg/ml)	1 ml
Amphotericin B (40mg/ml)	1 ml
Foetal calf serum	100 ml
HEPES, 1 M (N-2 Hydroxyethylpiperazine-N' 2 ethanesulfonic acid)	20 ml.
Distilled water to make	1000 ml

1) Add the constituents to distilled water and mix well. Adjust the pH of the medium to 7.4.

2) Sterilise by filtration.

3) Store the medium at 4°C.

Haemophilus ducreyi

1) ENRICHED GC AGAR

Used for *H. ducreyi* culture, it consists of:

- 1) GC agar base (formula per litre)

Peptone	15 gm
Corn starch	1 gm
Di-potassium hydrogen phosphate	4 gm
Potassium dihydrogen phosphate	1 gm
Sodium chloride	5 gm
Agar	10 gm
- 2) 1% Bovine Haemoglobin
- 3) 1% Isovitalax or CVA enrichment (Formula per 10 ml, when reconstituted)

Diphosphopyridine nucleotide (Coenzyme I)	2.5 mg
Coccarboxylase	1.0 mg
p-aminobenzoic acid	0.13 mg
Thiamine HCL	0.03 mg
Vitamin B 12	0.1 mg
L-Glutamine	100 mg
L-Cysteine	11.0 mg
L-Cysteine HCL	259.0 mg
Adenine	10.0 mg
Guanine HCL	0.3 mg
Ferric Nitrate	0.2 mg
Dextrose	1.0 mg
- 4) 5% Foetal Calf Serum
- 5) 3 mg Vancomycin per litre

Procedure:

- 1) Dissolve 36 gms of GC base in 450 ml of distilled water.
- 2) Boil the mixture.
- 3) Dissolve 10 gm of haemoglobin in 500 ml of distilled water separately.
- 4) Boil the mixture.
- 5) Autoclave both the mixtures at 115°C for 20 minutes.
- 6) Cool to 50-55°C.
- 7) Mix 10 ml of Isovitalax or CVA solution, 3 ml of 100 mg % Vancomycin solution and 50 ml of foetal calf serum with the GC base under strict aseptic precautions.
- 8) Mix the hemoglobin solution with the GC medium.
- 9) Mix and pour 14 ml of medium in a Petri dish of 90 mm diameter.

10) Pour multiple plates and store in refrigerator after the medium cools.

2) ENRICHED MUELLER HINTON CHOCOLATE AGAR

Used for *H. ducreyi* culture, it consists of:

1) Mueller Hinton agar (formula per litre)

Beef extract	2.0 gm
Acid hydrolysate of Casein	17.5 gm
Starch	1.5 gm
Agar	17.0 gm

- 2) 5% Horse blood.
- 3) 1% Isovitalax or CVA (refer Enriched GC Agar)
- 4) 5% Foetal Calf Serum (FCS).
- 5) 3 mg Vancomycin per litre.

Procedure:

- 1) Dissolve 38 gm of Mueller Hinton agar in 900 ml of distilled water.
- 2) Boil the mixture.
- 3) Autoclave the mixture at 115° C for 20 minutes.
- 4) Cool the solution in water bath to 80° C.
- 5) Add 50 ml of horse blood, mix thoroughly and immediately cool to 50-55° C.
- 6) Mix 10 ml Isovitalax or CVA solution, 3 ml of 100 mg % vancomycin solution and 50 ml of FCS with this solution.
- 7) Mix well and pour 14 ml of medium in sterile Petri dish of 90 mm diameter under strict aseptic precaution.
- 8) Pour multiples plates and store in refrigerator after the medium cools.

3) PHOSPHATE BUFFER

Prepare 0.025 M phosphate buffer, pH 6.8 as follows:

Solution A

KH_2PO_4	3.14 gms
Distilled water	1000 ml.

Solution B

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	4.46 gm
Distilled water	1000 mnl.

Prepare 100 ml of phosphate buffer by mixing 50.8 ml of solution A and 49.2 ml of solution B.

4) MUELLER HINTON AGAR WITH LYSED BLOOD, FOETAL CALF SERUM AND HD SUPPLEMENT.

Ingredients:

- 1) Mueller Hinton II agar (formula per litre)

Beef extract	2.0 gm
Acid hydrolysate of casein	17.5 gm
Starch	1.5 gm
Agar	17.0 gm
- 2) 5 % Horse blood by freezing and thawing.
- 3) 5% Foetal Calf serum.
- 4) 1 % of HD supplement (containing 10 gm glucose, 1 gm L-glutamine and 2.5 gm L-Cysteine hydrochloride per 100 ml of distilled water).

Procedure:

Prepare the complete medium in a similar fashion as explained in Enriched Mueller Hinton Chocolate Agar.

5) TRYPTICASE SOY BROTH

Ingredients:

Digest of Casein	17.0 gm
Soya peptone	3.0 gm
Sodium chloride	5.0 gm
Dipotassium hydrogen phosphate	2.5 gm
Dextrose	2.5 gm
Distilled water	1000 ml

Procedure:

- 1) Mix 30 gm of solid with 1000 ml of distilled water.
- 2) Distribute an amount of 2 ml in small tubes.
- 3) Close the tube with cotton stopper.
- 4) Autoclave at 115° C for 20 min.
- 5) Store in refrigerator after the tubes cool down.

6) BRAIN HEART INFUSION BROTH

Available commercially as dehydrated media. Reconstitute as per manufacturer's instructions.

- 1) Autoclave the reconstituted media at 121° C for 20 min. Store at 4° C in a refrigerator.
- 2) Distribute in sterile test tubes with cotton wool stoppers just before use.

Herpes simplex virus 1 and 2 (HSV-1 & 2)

1) TRANSPORT MEDIUM (VIRAL)

Ingredients:

Hanks balanced salt solution(HBSS)	900 ml
Bovine serum albumin 10 %	100 ml
Gentamicin	50 mg
Amphotericin	5 mg
Sodium bicarbonate at 7.5 % to adjust pH at 7.3.	

HANK'S BALANCED SALT SOLUTION:

Hanks balanced salt solution (HBSS) consists of :

Stock solution A

- | | | |
|----|--------------------|---------|
| 1. | Sodium chloride | 160 gms |
| | Potassium chloride | 8 gms |
| | Magnesium sulphate | 2 gms |
| | Magnesium chloride | 2 gms |
| | Distilled water | 800 ml |
-
- | | | |
|----|------------------|---------|
| 2. | Calcium chloride | 2.8 gms |
| | Distilled water | 100ml |
- 1) Mix the two solutions slowly.
 - 2) Make up the final volume to 1000 ml by adding distilled water.
 - 3) Add 2 ml chloroform and store at 4° C.

Stock Solution B

Disodium hydrogen phosphate	3.04 gms
Potassium di hydrogen phosphate	1.2 gms
Glucose	20 gms
Distilled water	800 ml

- 1) Dissolve the chemical in distilled water.
- 2) Add 100 ml of 0.4 percent phenol red in NaOH.
- 3) Make the final volume to 1000 ml with distilled water.
- 4) Store at 4° C after adding 2 ml chloroform.

For Use

Stock solution A	100 ml
Stock solution B	100 ml
Distilled water	800 ml

- 1) Dispense 100 ml in bottles.
- 2) Sterilize by autoclaving at 121° C for 20 min.
- 3) Store at 4°C.

Working Transport Medium

- 1) Take 900 ml of autoclaved HBSS.
- 2) Add rest of the contents to it and adjust pH at 7.3.
- 3) Dispense in small aliquots under strict aseptic precautions.
- 4) Store in refrigerator till use.

3) CULTURE MAINTENANCE MEDIUM (HERPES)

It consists of :

- Eagle's Basal Medium
- Eagle's Salt Solution
- L-Glutamine
- Foetal Calf Serum
- 20mM HEPES (N-2 hydroxyethylpiperazine-N'-2-ethane sulphonic acid)
- Or 8% NaHCO₃
- Gentamicin
- Amphotericin B
- Tryptose phosphate broth

Preparation of the medium:

Earle's Salt Solution (Commercially available)	70 ml
Eagle's basal medium (Commercially available)	8 ml
Tryptose phosphate broth (Oxide)	10 ml
Foetal calf serum-heated	2 ml
Gentamicin	5 mg
Amphotericin B	0.5 mg
NaHCO ₃ 8%	1 ml

Prepare the complete medium by mixing all the constituents under strict aseptic precautions.

Candida spp.

1) SABOURAUD'S DEXTROSE AGAR

Ingredients:

Dextrose	40.0 gm
Neopeptone	10.0 gm
Agar	15.0 gm
Distilled water	1000 ml

Procedure:

- 1) Mix the ingredients in distilled water by boiling. Adjust pH to 5.6.
- 2) Sterilize by autoclaving at 115° C for 15 min.
- 3) Allow to cool to 50°C.
- 4) Add Chloramphenicol 1 mg/ml of medium under aseptic precautions.
- 5) Pour 20 ml amounts in 90mm Petri dish or 10 ml into 15 ml test tubes for slants.
- 6) Allow the test tubes to rest at an angle so that agar slopes (slants) are obtained.
- 7) After the medium solidifies, keep the Petri dish and cotton wool stopped dextrose agar slants in the refrigerator.

2) CORNMEAL TWEEN-80 AGAR.

It is made up of Cornmeal agar, tryphan blue and Tween 80.

a. Cornmeal agar

Cornmeal (yellow)	40 gms
Agar	15.0 gms
Distilled water	1000 ml

Procedure:

- 1) Boil the cornmeal in 1 litre of distilled water for 60 minutes.
- 2) Filter through a filter paper or muslin.
- 3) Add the agar powder to the filtered solution.
- 4) Dissolve the agar by heating in a steam sterilizer.
- 5) Sterilize by autoclaving at 115°C for 20 minutes.

b) Preparation of complete medium.

- 1) Add 1 ml of tryphan blue (1% solution) and 10 ml of Tween-80 to 1 litre of cornmeal agar after boiling.
- 2) Sterilize by autoclaving at 115° C for 15 minutes.
- 3) Pour into sterile Petri dishes (15 ml/plate).

3) BASAL MEDIUM AGAR (For Candida Sugar assimilation test)

Agar	20.0 gm
Bromocresol purple base	0.020 gm
Yeast nitrogen base	0.67 gm
Distilled water	1000 ml

- 1) Suspend the solid components in distilled water.
- 2) Boil the solution to melt the agar.
- 3) Adjust the pH to 7.0 by adding 1 N NaOH.

- 4) Distribute in screw capped bottles and autoclave at 121° C for 15 minutes (Leave the caps loose during the procedure).
- 5) Allow the medium to cool, tighten the screw caps and store at 4° C in a refrigerator. The medium should be purple and can be stored for several months

CARBOHYDRATE SOLUTION FOR ASSIMILATION

The recommended quantity of each sugar to be placed in each quadrant is as follows:

Inositol	2-4 mg
Dextrose	3-6 mg
Maltose	5-7 mg
Raffinose	10-14 mg
Cellobiose	10-14 mg
Melibiose	15-20 mg

Procedure:

- 1) Make 100 mg/ml stock solutions of the sugars.
- 2) Sterilize by Seitz Filtration and store in sterile bottles with droppers.
- 3) Find the number of drops delivered per ml of the solution. This gives the amount of sugar delivered per drop.
- 4) Accordingly place the requisite number of drops in each quadrant.
e.g 1 drop = 0.05 ml of volume.
Melibiose stock solution has a concentration of 100 mg/ml. So one drop will have to be superimposed on the same spot in the particular quadrant of the plate to give the concentration of 16-20 mgs melibiose/quadrant.
- 5) Alternatively, prepare the stock solution in such a concentration that each drop gives the required milligrams of the carbohydrate.
e.g 1 drop = 0.05 ml.
Melibiose will have to be made in a concentration of 400 mg/ml because then 1 drop will deliver 400 mg/ml x 0.05 ml = 20 mg to the single test quadrant.

Trichomonas vaginalis

1) DIAMOND'S TRICHOMONAS MEDIUM

Ingredients:

Trypticase	20.0 gm
Yeast extract	10.0 gm
Maltose	5.0 gm
L-Cysteine hydrochloride	1.0 gm

L-Ascorbic acid	0.2 gm
Di-potassium hydrogen phosphate	0.8 gm
Potassium dihydrogen phosphate	0.5 gm
Agar	0.5 gm
Distilled water	900 ml

Procedure:

- 1) Mix the contents in distilled water.
- 2) Autoclave the medium at 115°C for 20 minutes.
- 3) Cool the medium to 50°C and add 100 ml sheep or bovine serum (sterilized by filtration) and 10 mgs % Chloramphenicol.
- 4) Store at 4°C till use.

2) KUPFERBERG TRICHOMONAS MEDIUM

Ingredients:

- 1) Kupferberg trichomonas broth base medium
- 2) Beef or human serum
- 3) Trichomonas selective supplement

Procedure:

Suspend 23.50 gm of Kupferberg trichomonas broth base medium in 950 ml of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C, 15 lb. pressure for 15 minutes. Cool to 50-55°C and aseptically add 50 ml of sterile beef or human serum and rehydrated contents of Trichomonas selective supplement.

3) WHITTINGTON TRICHOMONAS MEDIUM

Ingredients:

- | | |
|----------------------|------------------|
| 1) Dextrose | 5.0 gm |
| 2) Sodium chloride | 6.5 gm |
| 3) Proteolysed liver | 2.0 gm |
| 4) Distilled water | 1000 ml (pH 6.5) |

Procedure:

Mix 5 gm of dextrose, 6.5 gm of Sodium chloride and 2 gm of proteolysed liver in one litre of distilled water. Sterilize by autoclaving at 121°C, 15 lb. pressure for 15 minutes. Cool to 50-55°C and aseptically add 80 ml of horse serum, 1 gm of benzyl penicillin and 5 gm of streptomycin. Pour 5 ml medium into each tube.

4) **MODIFIED COLUMBIA AGAR (MCA)**

Dissolve 13 g of Columbia agar (Oxoid Ltd.) in 320 ml of distilled water

Adjust to a pH of 6

Boil for 20 min

Autoclave at 121°C for 5 min, and cool

Add 50 ml of sterile, inactivated lamb serum (PAA Laboratories) containing 4 g of dextrose (Sigma Chemical Co.), 4 g of malt extract (Oxoid Ltd.), 250 U of bacitracin/ml and 5,000 U of neomycin/ml; 10 ml of nystatin solution (10,000 U/ml; 4 ml of penicillin-streptomycin solution (10,000 of penicillin/ml and 10,000 µg of streptomycin/ml; and 1 ampoule of chloramphenicol selective supplement (Oxoid Ltd.).

Pour 10 ml of the resulting mixture into 6-cm-diameter petri dishes.

5) **TYM MEDIUM**

20 gms Trypticase (casein digest peptone)

10 gms Yeast extract

5 gms Maltose

1 gm L cysteine hydrochloride

0.2 gms Ascorbic acid

0.8 gms Potassium phosphate dibasic

0.8 gms Potassium phosphate monobasic

1. Dissolve the above in 600ml Distilled water

2. Adjust pH to 6

3. Bring to 900 ml of Distilled water

4. Dispense in 90 ml amounts

5. Autoclave for 15 minutes at 121°C under 15 lb/square inch

6. On cooling add 10 ml of heat inactivate of bovine serum

7. Can be stored at -20°C

8. To improve isolation, add 0.5 gm of Bacto agar to the medium.

6. **HOLLANDER'S MODIFICATION OF TY MEDIUM**

20 gms Trypticase (casein digest peptone)

10 gms Yeast extract

5 gms Maltose

1 gm Ascorbic acid

1 gm Potassium chloride

1 gm Potassium bicarbonate

1 gm Potassium phosphate monobasic

0.5 gms Potassium phosphate dibasic

0.1 gm Ferrous sulphate

1. Dissolve the above in 600ml Distilled water
2. Adjust pH to 6
3. Bring to 900 ml of Distilled water
4. Dispense in 90 ml amounts
5. Autoclave for 15 minutes at 121°C under 15 lb/square inch
6. On cooling add 10 ml of heat inactivate bovine serum
7. To improve isolation, add 0.05% of Bacto agar to the medium

Add the following in the above culture media

Vancomycin 0.02 gms/lit

Gentamycin 0.02 gms/lit

7. DEHYDRATED TRICHOMONAS MEDIUM of Difco (Composition directions for use as per the bottle instructions) -A medium based on that of Feinberg & Whittington for the detection of *Trichomonas vaginalis*.

Liver digest 25gm/l

Glucose 5gm/l

Sodium chloride 6.5 gm/l

Agar 1 gm/l

pH 6.2

- ▶ Suspend 37.5g of the above in 1 litre of distilled water
 - ▶ Boil to dissolve.
 - ▶ Sterilise by autoclaving at 121°C for 15 minutes.
 - ▶ Cool to approximately 50°C.
- Inactivate 80ml of horse serum and add to the medium along with 1000 units of penicillin and 500µg of streptomycin per ml of medium or 100µg of chloramphenicol per ml of medium.

8. TY1-S 33 MEDIUM

- ▶ 1 gm Potassium phosphate dibasic
 - ▶ 0.6gm Potassium phosphate monobasic
 - ▶ 20 gms Trypticase (casein digest peptone)
 - ▶ 10 gms Yeast extract
 - ▶ 10 gms Glucose
 - ▶ 1 gm L cysteine hydrochloride
 - ▶ 0.2 gms Ascorbic acid
 - ▶ 1 ml Ferric ammonium citrate
1. Dissolve the above in 600ml Distilled water
 2. Bring to 880 ml of Distilled water

3. Adjust pH to 6
4. Dispense in 88 ml amounts in 125 ml glass bottles
5. Autoclave for 15 minutes at 121°C under 15 lb/square inch

ANNEXURE - III

GUIDELINES FOR SAMPLE AND PATIENT TRANSFER

I. Sample transfer

A. Gonorrhoea

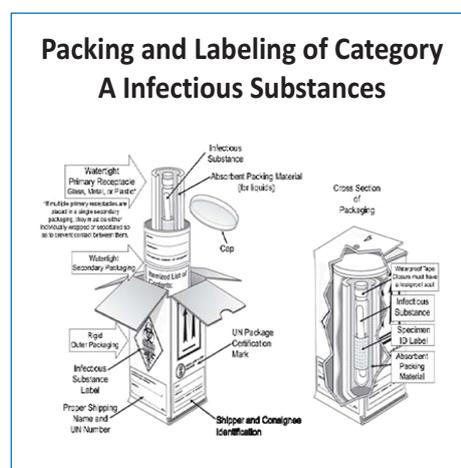
- ▶ Smear for Gonococci to be stained by Gram's stain and examined at STI Clinic. For confirmation at a reference laboratory, it may be wrapped individually in aluminium foil and packed in slide boxes and transported.
- ▶ Request slip- Name, age, sex, risk factors, H/o previous test etc. Place in plastic pouch with zip lock.
- ▶ Samples to be collected for culture and sensitivity tests- urethral swab/ endocervical swab/ rectal swab/ pharyngeal swab depending on symptoms. To be transported in Stuart's or Amie's transport media within 24 hours. If longer delay is anticipated, then Transgrow media or Jembec media may be used. However, these media are expensive.

B. Chlamydia

1. Samples to be collected for DFA and ELISA for antigen detection- Urethral swab/ endocervical swab/ rectal swab/ bubo pus depending on symptoms. Swabs may be transported in transport media provided along with the kits.
2. Sera for antibody detection by ELISA- Collect 3-5 ml blood in screw capped plastic plain tubes. Allow to clot and separate sera. Transport the sera in screw capped, 2 ml plain Eppendorf vials.
 - ▶ Specimen tube must not have cracks/ leaks
 - ▶ Disinfect the outside after tightly capping.
 - ▶ Place in leak proof container (eg. sealed plastic pouch with zip lock/ stapled and taped)
 - ▶ Pack this inside a cardboard canister/ box with sufficient cotton gauze or other absorbent material to absorb all leaked serum.
 - ▶ Cap the canister/box tightly
 - ▶ Fasten the request slip securely to outside of the box.
 - ▶ Request slip- Name, age, sex, risk factors, H/o previous test etc. Place in plastic zip lock.
 - ▶ For mailing- this canister/box must be placed inside another box with mailing label and biohazard sign.

C. Syphilis

Sera for confirmatory test (TPHA/ FTA-Abs /ELISA) to be transported as described above for Chlamydia.



- D. Chancroid
- ▶ Samples to be collected for culture and sensitivity- Ulcer swab may be collected and transported in Amie's transport media as described above.
 - ▶ Smear for *H. ducreyi* to be stained by Gram's stain and examined at STI Clinic. For confirmation at a reference laboratory, it may be wrapped individually in aluminium foil and packed in slide boxes and transported.
 - ▶ Request slip- Name, age, sex, risk factors, H/o previous test etc. Place in plastic pouch with zip lock.
- E. Granuloma inguinale (Donovanosis)
- Smear for Donovan body to be stained by Giemsa stain and examined at STI Clinic. For confirmation at a reference laboratory, it may be sent as above.
- F. Herpes progenitalis
- ▶ Smear for Multi nucleated giant cells (MNGC) to be stained by Giemsa stain and examined at STI Clinic. For confirmation at a reference laboratory, it may be sent as above.
 - ▶ Ulcer swab may be collected in the transport media supplied with the ELISA kit for antigen detection, and transported to the reference laboratory.
 - ▶ Sera for antibody detection by ELISA may be transported as described above.
- G. Candidiasis
- ▶ Vaginal smear for budding yeast cells and pseudohyphae to be stained by Gram's stain and examined at STI Clinic. For confirmation at a reference laboratory, it may be sent as above.
 - ▶ Vaginal swab for culture must be collected in a sterile tube containing 0.3 ml saline and transported by maintaining appropriate cold chain.
- H. Trichomoniasis
- Vaginal swab for culture must be collected in a sterile tube containing Amie's transport media and transported within 24 hours.
- I. Bacterial vaginosis
- Vaginal smear for clue cells, to be stained by Gram's stain and examined at STI Clinic by Nugent's criteria. For confirmation at a reference laboratory, it may be sent as described above.
- J. Human Papilloma virus infection
- Collect the specimen (exfoliated cells) with a sterile cotton wool swab soaked in saline, or wooden /plastic spatula. In females, cervical samples from the junctional zone between exo and endo cervix to be collected. Transported to reference laboratory for Papanicolaou stain as described above.
- K. Hepatitis B virus
- Sera for HBsAg detection to be collected and transported as described earlier.
- L. Hepatitis C virus
- Sera for anti HCV antibody detection to be collected and transported as described earlier.
- II. Patient transfer**
- Rarely required. When necessary, follow general rules of patient transfer.

ANNEXURE - IV

STANDARDS FOR STI / RTI LABORATORY SERVICES AT REGIONAL STI CENTRES (RSC)/ STATE REFERENCE CENTRES (SRC)

Quality STI/RTI laboratory Service Delivery

- i. All RSC/SRC - should provide defined package of quality services for prevention, diagnosis and management of STI/RTIs.
- ii. RSC/SRC - should have adequate infrastructure in terms of space, human resources, equipment, furniture, reagents and consumables.
- iii. RSC/SRC - should have provision for periodic training of staff in delivery of quality services.

Components of Quality STI/RTI laboratory Service Delivery

- i. Location
- ii. STI/RTI service package
 - a. Counselling service
 - b. Clinic (male and female) service
 - c. STI laboratory & diagnostic facility- RSC /SRC
 - d. STI treatment facility
 - e. Training facility in STI/RTI at RSC /SRC

Infrastructure for RSC/SRC

- i. Equipment
- ii. Human resources
- iii. Office equipment & furniture
- iv. Kits, media and reagents
- v. Consumables
- vi. Patient records, referrals and reporting
- vii. Internal quality control & EQAS
- viii. Biomedical waste disposal system
- ix. Infection control system
- x. Condom distribution

TOOLS FOR EVALUATING STI/ RTI LABORATORY SERVICES AT REGIONAL STI CENTRES(RSC)/ STATE REFERENCE CENTRES (SRC)

i. Location

State:

District:

Name of Centre:

Complete address:

Name and Phone Number of Service Provider:

Unique ID Number :

ii. Facilities uptake and service package

Is the STI Centre diagnosing and treating all possible STI/RTI cases coming to the hospital?

iii. Infrastructure for RSC /SRC

1. Waiting area
2. Laboratory Diagnostic area- for processing and test performance
3. Specimen Collection area
4. Washing facility
5. Sterilization area
6. Packing/swab making area
7. Media preparation area
8. Store room
9. Office area
10. Toilets (male and female)

iv. Availability of equipment-

1. Binocular bright field microscope
2. Binocular dark field microscope
3. Binocular fluorescent microscope
4. ELISA reader, printer, washer
5. Biosafety cabinet /Laminar air flow hood
6. VDRL rotator
7. Incubator
8. BOD incubator
9. Hot Air Oven
10. Autoclave
11. Deep freezer (-20°C)
12. Deep freezer (-80°C)
13. Centrifuge

14. Serological water bath
15. Vortex machine
16. Digital/Electronic balance
17. pH meter
18. Micropipettes of various capacities.
19. Water Distillation plants
20. Lyophiliser (optional)
21. Teaching microscope
22. Needle destroyers
23. PCR (optional)
24. Mobile STI van (optional)

v. Availability of human resources

1. Qualified and dedicated venereologist (male/female)
2. STI Microbiologist
3. Epidemiologist
4. Dedicated staff nurse
5. Counsellors (male and female)
6. Laboratory supervisor
7. Laboratory technician
8. Laboratory assistants
9. LDC/UDC
10. Lady health visitor (LHV)/MHV
11. Health educator
12. Store keeper
13. Nursing attendants
14. Safai karamchari
15. Driver
16. Designated quality manager
17. Designated safety officer

vi. Availability of office equipment and furniture

1. Display plates and boards for all rooms
2. Photocopier
3. Computer-(PC with printer, DVD writer (optional), scanner) /Laptop
4. Fax machine (optional)
5. Telephones (PABX and EPABX)
6. LCD facility
7. Almirahs
8. Revolving stools

9. File cabinets
10. Refrigerators
11. General office furniture (tables/chairs)

vii. Availability of kits, media & reagents

1. Reagents for Gram's stain
2. Reagents for Giemsa stain
3. Chemicals for wet mounts (KOH), buffers etc.
4. Media for GC culture
5. Media for Candida culture
6. Media for *H. ducreyi* culture
7. Media for *T. vaginalis* culture
8. Media for culture of pyogenic organisms
9. Kits for Syphilis- VDRL/ RPR, TPHA, FTA-Abs, ELISA
10. Kits for Chlamydia- DFA, ELISA (both antigen & antibody)
11. Kits for Herpes simplex-2-ELISA (both antigen & antibody)
12. Kits for HBsAg detection
13. Kits for anti HCV antibody detection

viii. Availability of consumables

1. Disinfectants
2. Gloves
3. Colour coded waste disposal bags.
4. Syringes
5. Needles
6. Vacutainers
7. Specimen collection receptacles
8. Sterile swabs
9. Glassware and plastic ware

ix. Availability of patient records, referrals & reporting

1. Complete and correct patient wise record
2. Laboratory registers (test-wise)
3. Investigation requisition proforma
4. Clinic register (Male/Female)
5. Storage cupboards/almirahs with lock and key for register
6. Monthly report proforma
7. Counsellors registers
8. Consent forms wherever applicable
9. Equipment stock register
10. Equipment log book

11. Indent registers
12. Equipment condemnation register
13. Occurrence and accident register
14. Suggestion/ complaint register
15. EQAS record registers

x. Availability of Internal Quality Control (IQC) and EQAS

1. Staff member designated for IQC ad EQAS

xi. Availability of Biomedical waste disposal system

1. Colour coded bags
2. Colour coded containers
3. Needle destroyers
4. Dedicated autoclave for waste disposal.
5. Microwave
6. Disinfectants
7. Heavy duty gloves
8. Spills kit
9. Posters for education
10. Training and refresher course material

xii. Infection control system in place

1. Barrier protection devices in place (gloves, masks, eye shields, boots)
2. Hand washing materials
3. Spills kit
4. Waste disposal system.
5. Eye wash facility

xiii. Availability of condom distribution system

1. Good quality Govt. supply of condoms
2. Educative pamphlets
3. Penis model

CHECK LIST

1. Laboratory performance
2. Laboratory staffing
3. Microbiologist performance
4. Lab. technician performance
5. Clinician performance
6. Counsellor performance

7. Nurse performance
8. Class III and IV performance
9. Completeness of patient records
10. Laboratory records
11. Laboratory equipment maintenance
12. Media, kits and consumables
13. Referral network
14. Infection control system
15. Ethical standards and confidentiality
16. Training and refresher/reorientation course activities.
17. EQA and IQC

TOOLS FOR EVALUATING STANDARDS FOR STI/ RTI LABORATORY SERVICES AT STI/ RTI CLINICS

The STI/RTI Clinics in the periphery should be equipped to perform following simple diagnostic procedure:

- i. **Syphilis screening** – RPR test /VDRL if feasible.
- ii. **Wet Mount** – for *Trichomonas vaginalis*, *Candida* spp., Bacterial vaginosis.
- iii. **Giemsa staining** – for Donovan bodies, Multi nucleated giant cells (MNGC).
- iv. **Gram staining** – for *Candida*, Bacterial vaginosis, *Haemophilus ducreyi*, *Neisseria gonorrhoeae*.
- v. **pH test** – for Bacterial vaginosis.
- iv. **Whiff test** – for Bacterial vaginosis

ANNEXURE - V

BACTERIOLOGY

Introduction

The isolation and identification of bacteria causing sexually transmitted infections is accomplished by several techniques in the laboratory. A single test is often inconclusive and hence, many tests may have to be performed in order to arrive at a correct diagnosis. These techniques include-

- a. Direct examination of bacterial smears- By wet mounts or stained smears. This is generally not sufficient to identify the bacterial species, although it can give a fair idea about the type of bacteria to expect and media to be used for culture. This shows the importance of proper collection and dispatch of specimens to referral laboratories. Nevertheless, direct microscopic examination of stained smears is an efficient way of studying the presence of bacteria in biological fluids that are normally sterile, such as cerebrospinal fluid (CSF) and pleural fluid, and in specimens from other sources. It may provide information of great value for the diagnosis, immediate treatment and control of the disease in limited cases. For eg.--
 - I. The value of direct smears may be high in specimens from cases of male urethritis, as an early stage gonococcal infection can be diagnosed with reasonable certainty (in females it is much more difficult).
- b. Culture of bacteria in artificial media- When conditions appropriate to the growth of bacteria and similar to the human body milieu can be provided in the laboratory, in the form of artificial media in Petri dish or test tubes, then it is possible to isolate the bacteria in the laboratory. Eg. It is possible to culture *Neisseria gonorrhoeae* and *Haemophilus ducreyi* in the laboratory.
- c. Biochemical tests on the isolated cultures- Performing certain biochemical tests will often bring out certain unique properties of bacteria and thus aid in the accurate species level diagnosis.
- d. Serological tests- The diagnosis of some diseases is also possible through serology, especially when culture of the bacteria is not feasible. Eg. Syphilis. Serological techniques are also important for sero epidemiological surveillance.

Principle

The sample to be examined (pus, urethral/cervical/vaginal discharges, urine centrifugate, cerebrospinal fluid, etc.) is treated as follows:

- ▶ It is spread in a thin layer on a glass slide
- ▶ It is dried completely
- ▶ It is fixed on the slide by gentle heating before being stained.

MATERIALS

Inoculating loop



Inoculating loop

This is a metal wire (usually made of nickel-chromium alloy-Nichrome) fixed on to a handle and bent into a loop at the other end. Make the loop with forceps, taking care that it is centred.

Figure 1. Inoculating loop

Clean with an ethanol ether mixture and wipe with gauze



Figure 2. Glass slide



Bunsen Burner - Should have a blue flame for optimal use.

Figure 3. Bunsen burner

Preparation of smear
Hold the loop just above the blue part of the flame
Hold the instrument as nearly vertical as possible.
Flame the loop until it is red-hot
Allow to cool (count to 20)



Figure 4. Flaming



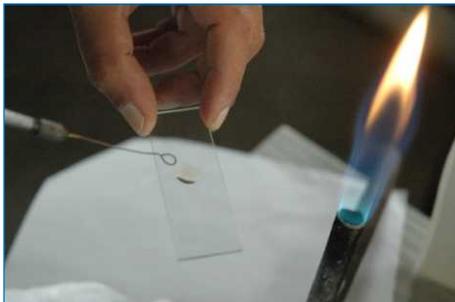
Take a portion of the specimen to be examined for pus, by placing the loop flat on the surface of the liquid. Fig. 5 & 6

Figure 5. Smear making-step-1

Remove a loopful of material taking care not to touch the sides of the test tube.



Figure 6. Smear making - step-2



Place the loop on the slide and press slightly flat and in the center (the slide should be numbered) (Fig.7). Make a smear using a swab or loop.

Figure 7. Smear making step-3

1. Still holding it flat against the slide, move the loop in an overall, spiral, out wards from the center.
2. Leave a space between the specimen and each of the 4 sides of the slide. (Fig. 8).



Figure 8 Smear making step-4



Let the slide dry completely in air. Label the slide with a glass maker pen/ pencil. (Fig.9)

Figure 9. Labelling the slide



Unmarked smears are sometimes received in the laboratory from outside sources. To find out on which side of an unmarked slide the smear has been made, turn the slide so that it reflects the light from the window. The side without the smear will shine. The side with the smear will not reflect the light and remain dull. (Fig. 10)

Figure 10. Smear side differentiation.

Fixation

The smear has to be fixed on the slide. (Fig 11). Check that the smear is completely air-dried. Pass the slide through the flame of a Bunsen burner, with the smear uppermost. Pass it through the flame three times in quick succession. Allow to cool before staining.

It is sometimes useful to draw a circle around the smear with a grease pencil, so that it can be seen more easily.



Figure 11. Fixing of smear.

STAINING OF FIXED SMEARS

Gram stain, (described elsewhere in this manual) Reading of stained direct smears, (described earlier).

Gram Staining

Gram stain- is a differential staining technique, described first by Hans Christian Gram in 1884.

Advantages-

Gram staining makes it possible to classify bacteria into two groups:

1. Gram positive bacteria- stain dark violet.
2. Gram negative bacteria- stain pink.

This makes identification easier.

Gram staining reagents :

Primary stain- Crystal violet/Gentian violet

Gram's iodine, solution 95% ethanol, Safranin solution and tap water.



Figure 12. Staining reagents

Technique:

Fix the smear and allow cooling. Crystal violet- Pour the crystal violet on to the slide. Cover the slide completely. Leave for 1 minute. Rinse with tap water and drain.

Gram's iodine solution – flood the slide with Gram's iodine solution. Let it stand for 1 minute. Drain off the solution and rinse with tap water.

Decolourising

95% ethanol- 10-15 secs.

Cover the slide completely. Leave for 10-15 secs. Flood with water and drain.

Look at the smear:

If violet patches remain, treat again with ethanol for 15 to 30 seconds. Rinse well with water and drain.(Fig.13)

Safranine solution – 30 seconds

Leave on the slide for 30 seconds Wash briefly with water at once.

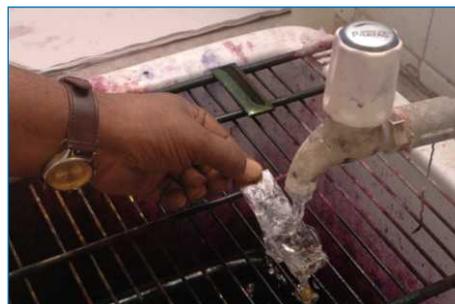


Figure13. Gram staining procedure- (decolourising)

Draining

Drain and allow to dry in the air. (Fig.14)



Figure 14. Gram's staining procedure (draining)

WHAT TO LOOK FOR

Bacteria stained deep violet: Gram positive, e.g.

Staphylococci, Streptococci, Micrococci, Pneumococci, Enterococci, Diphtheria bacilli, anthrax bacilli.

Bacteria stained pink: Gram negative, e.g. Gonococci, Meningococci, coliform bacilli, Shigellae, Salmonellae, Cholera vibrio.

PRINCIPLE OF THE STAINING REACTION

1. In the first step, the violet colour stains all bacteria deep violet.
2. Gram's Iodine solution is a mordant and fixes the violet colour more or less strongly in the bacteria
3. 95% ethanol:
 - ▶ Decolourizes certain bacteria when the violet stain is not strongly fixed by iodine solution
 - ▶ Does not decolorize the bacteria when the violet stain is strongly fixed by iodine solution.
4. Safranine solution (pink):
 - ▶ Re-stains (pink) the bacteria discoloured by ethanol
 - ▶ Has no effect on the other bacteria, which remain dark violet.

QUALITY CONTROL

A known gram positive and a gram negative stain should be used as quality control;

S. No.	Control Organism	Expected Result
1	<i>S.aureus</i> ATCC 25923	Gram positive cocci
2	<i>E.coli</i> ATCC 25922 <i>Peudomonas aeruginosa</i> ATCC 27853	Gram negative bacilli

SOURCES OF ERROR

A false Gram positive reaction may occur because:

- ▶ The smear was fixed before it was dry.
- ▶ The smear was too thick .
- ▶ There was sediment in the bottle of crystal violet (always filter before using).
- ▶ The Gram's iodine solution was not thoroughly drained off.
- ▶ The ethanol was not left long enough.
- ▶ The violet stain was too strong or left too long on slide.

A false Gram negative reaction may occur because:

- ▶ The safranin solution was too strong or left on the slide too long
- ▶ The Gram iodine solution was not left long enough.
- ▶ The ethanol was left on too long and not washed off properly.

Bacteria are 0.1- 0.5u x 3-7 u in size and can only be visualized under a microscope or isolated in artificial culture media when they form colonies.

A distinction is made between the following types of bacteria-

*Pathogenic bacteria**

These are capable of causing disease in man. They are found in the body they infect and are detected by laboratory methods.

Non-pathogenic bacteria

These are harmless bacteria that exist in countless numbers in nature. Some multiply normally in man without affecting his health and are known as commensals. Others are capable of causing infection and disease when the opportunity arises (like underlying diseases or immuno compromised status of host). Such bacteria are called Opportunistic pathogens.

HOW BACTERIA ARE DETECTED IN THE LABORATORY

1. **By direct examination of wet mounts** or hanging drop preparation- to look for motility and morphology.
2. **By direct microscopical examination** of smears (from specimens of pus, urine, sputum, skin, CSF, nose or throat) made on slides and stained (by Gram or Zeihl-Neelsen stains).
3. **By bacterial culture:** On solid culture media (agar in a Petri dish or test-tube) in liquid media (tubes of broth). Cultures are essential to determine the exact identity of bacteria and, more particularly, to determine whether the organisms found in the specimens are pathogenic or non-pathogenic. Biochemical, serological (agglutination) and other tests are used to identify the organisms cultured. Never fail to send specimens to a more specialized laboratory for culture whenever necessary (for dispatch of specimens see elsewhere in this manual).

*Note: Obligatory and facultative pathogens. Obligatory pathogens are those that always cause

disease (e.g. tubercle bacilli), facultative pathogens are harmless in certain areas of the body (e.g. coliform bacilli, a normal commensal of the intestine), but can cause disease when they invade other areas (the coliform bacillus may infect the urinary tract).

Direct examination is most useful in obtaining an indication of the type of organism involved or, in some cases, in establishing a diagnosis of the disease. Eg. Gonorrhoea. For this purpose it is essential to give a detailed description of the organisms seen as well as of any other elements present (leukocyte, red blood cells, epithelial cells etc.)

VARIOUS GROUPS OF BACTERIA SEEN UNDER THE MICROSCOPE (DIRECT EXAMINATION BY GRAM STAIN)

Gram positive cocci (Fig.15)– round shape
May be arranged:
▶ In clusters (Staphylococci)
▶ In chains (Streptococci)
▶ In pairs (Pneumococci)
▶ In fours or tetrads (Micrococci).
Found in pus, urine, blood and other specimens.

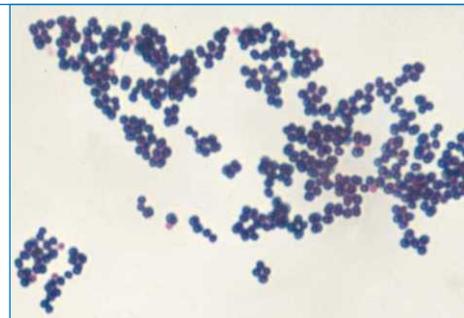


Figure 15 Gram positive Cocci

Gram negative diplococci (Fig.16) – rounded shape in pairs:
May be shaped like coffee bean cluster in the cytoplasm of the leukocyte. Found in urethral pus (gonococci) and the CSF (meningococci). There are other Gram negative diplococci that are generally non-pathogenic. They may be seen in throat swabs or sputum specimens.

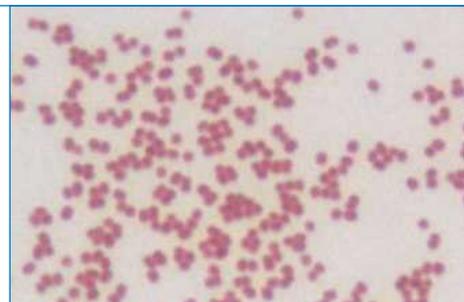


Figure 16 Gram negative diplococci

Gram positive bacilli (Fig 17) – rod shaped
(a) Gram positive bacilli- with spores
Long, thick and may have square ends (anthrax) or rounded ends (tetanus, saprophytes). The spore appears as a large uncolored area inside the bacillus as it does not stain with Gram stain.
(b) Gram positive bacilli- without spores. Usually small and variable in shape; the ends may be swollen and be arranged in rows or like letters. Found in throat specimens, blood, skin, etc. (Corynebacteria, Diphtheroids, Listeria).



Figure 17 Gram positive bacilli

Gram negative bacilli (Fig.18)

Variable in size, ends rounded or pointed. May be larger and straight (coliform bacilli), comma-shaped (*Vibrio*) or short and fat (*Klebsiella*). This group includes many species.



Figure 18. Gram negative bacilli

Gram negative coccobacilli (Fig.19)

Quite variable in shape, not as round as cocci but not as long as normal bacilli either (*Yersinia*, *Haemophilus*). Found in a variety of body specimens.

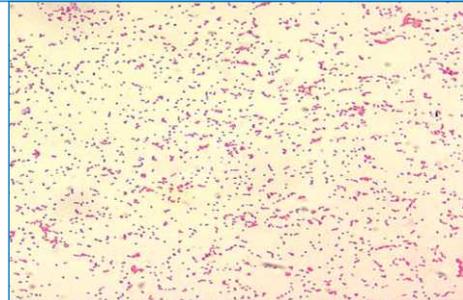


Figure 19. Gram negative coccobacilli

Yeasts (Fig.20)- Vary in size but are larger than bacteria. May be seen in the process of budding or as single cells. Usually present as contaminants but sometimes may be pathogenic. Eg *Candida* in genital discharge, sputum.



Figure 20 *Candida*

Actinomycetes (Fig.21) - Large granules, sometimes visible to the naked eye, white to yellow in color. The center is Gram negative, the periphery Gram positive. Found in pus from skin, sputum, etc.

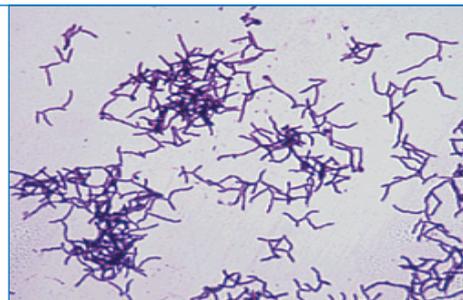


Figure 21 Actinomyces

Spirochaetes (Fig.22)- Eg *Treponema*, *Borrelia*
Regular loose spiral – weakly staining Gram negative.
Treponema vincentii (formerly *Borrelia vincenti*)
Found together with cigar-shaped Gram negative rods (fusiform bacilli) in throat and mouth.



Figure 22 Spirochaetes (Dark field microscopy)

Sterile Body Cavities, Fluids, and Tissues	Non-Sterile Body Cavities, Fluids and Tissues
<p>In healthy persons the following are sterile:</p> <ul style="list-style-type: none"> ▶ Blood ▶ Cerebrospinal fluid ▶ Subcutaneous tissue ▶ Internal organs (heart, liver, kidney, etc.) 	<p>In healthy persons following may contain many non-pathogenic organisms (commensals).</p> <ul style="list-style-type: none"> ▶ Respiratory tract (mouth, nose, throat, (sputum) ▶ Gastrointestinal tract ▶ Skin, ear and eye ▶ Urogenital tract (vagina, anterior urethra)
<p>In disease all of these areas may be infected by pathogenic organisms.</p>	

RECORDING THE RESULTS OF DIRECT BACTERIOLOGICAL EXAMINATION

The laboratory report must give a detailed description of all the elements and organisms found and their number.

Elements

Type:	leukocytes, red blood cells, epithelial cells.
Organisms:	
Shape	cocci, bacilli, etc
Arrangement	pairs, chains, clusters
Staining properties	Gram, Ziehl-Neelsen
Special Characteristics	Spores, granules, etc
Quantity	occasional, a few, a moderate number, many, plenty, profuse

Example of reports

- 1) Pus from abscess (direct bacteriological examination) (Gram stain):
 - ▶ Many leukocytes
 - ▶ A few red blood cells
 - ▶ A few epithelial cells
 - ▶ A moderate number of Gram positive cocci in clusters
- 2) Urine (direct bacteriological examination) (Gram stain):
 - ▶ A few leukocytes
 - ▶ Occasional red blood cells
 - ▶ A few epithelial cells
 - ▶ A few Gram negative bacilli
- 3) Sputum (direct bacteriological examination) (Ziehl-Neelsen)
 - ▶ 5 acid- fast bacilli found/10 fields (2+)

- 4) Throat specimens (direct bacteriological examination) (Gram stain):
- ▶ A few leukocytes
 - ▶ A few red blood cells
 - ▶ A few epithelial cells
 - ▶ Many Gram positive cocci in chains
 - ▶ A few Gram positive bacilli without spores (diphtheroids) arranged in V or L forms
 - ▶ A few Gram negative diplococci
 - ▶ Occasional Gram negative bacilli

Important:

It is rare to diagnose a disease in the laboratory on the basis of the identification of the organisms found by direct bacteriological examination of a specimen. The results of such an examination, however, can help the physician to establish a diagnosis when taken together with the symptoms shown by the patient.

Gonococci: Direct Examination of Urethral Pus

GONORRHOEA

This is one of the important STIs caused by *Neisseria gonorrhoeae*. The incubation period is 4 to 8 days. Typical symptoms include genitourinary discharge. Genitourinary discharges may occur in other diseases too, as mentioned below:

Thick yellow pus:	Likely to be Gonorrhoea
Clear whitish fluid, frothy and foul smelling:	Trichomoniasis
Thick, curd-like, cheesy-white exudate:	Fungus
Other exudates:	Non - specific urethritis (not identifiable by direct examination).

Principle -

Smears of urethral pus are stained with Gram stain. Gonococci can be recognized by three characteristics:

1. Diplococci (in pairs)
2. Gram negative
3. Intracellular (inside the leukocytes).

Collection of specimen from male

1. If possible, take the specimen first thing in the morning before the patient has urinated. If necessary, clean the meatus with a swab moistened with sterile sodium chloride solution.
2. Exert a slight pressure on the penis so that a drop of pus appears at the meatus.
3. Remove the pus with a sterile inoculating loop/swab.



Figure 23. Sterile swab

4. Apply directly to a clean slide (Fig.23).If no pus appears, insert the sterile loop approximately 2.5 cm up the urethral canal to obtain a specimen.
5. Prepare two smears that:
 - ▶ are as thin as possible
 - ▶ cover as much of the slide as possible.

Direct examination is of great value for the diagnosis of gonorrhoea in males; it is much less so in female. Culture is therefore necessary to isolate and identify the gonococci in specimens from females.

Staining the smears-

Stain with Gram stain. Treat thoroughly with ethanol after applying Gram iodine solution. Wash with water at once after final staining with safranin solution. Some prefer Neutral red to Safranin as a counter stain, for Gonococcus.

Examination of slides-

Pay particular attention to the edges of the smear, where the elements are spread more thinly and are easier to see and the stain is less concentrated.

Pus (Note whether there are many masses of degenerate leukocytes. The nuclei are bright pink and the cytoplasm is colorless).

Gonococci oval, kidney-shaped, Gram negative (pale pink) cocci

Evaluation of results of direct bacteriological examination of pus from urethra

Many leukocytes	Many leukocytes	Many leukocytes
A few red blood cells A few epithelial cells A moderate number of gram negative intracellular diplococci	A few red blood cells A few epithelial cells No gram negative Intracellular diplococci. Gram negative extracellular diplococci seen.	A few red blood cells A few epithelial cells No gram negative Intracellular diplococci Extracellular diplococci
CONCLUSION Gonococci-Positive	Gonococci-suspicion	Gonococci-negative

Other bacteria causing urethral infections

Male: A number of the following may occasionally be seen in smears of urethral pus:

- ▶ Gram positive cocci (Staphylococci)
- ▶ Gram positive bacilli (Diphtheroids)

- ▶ Gram negative bacilli

Never make a direct examination for gonococci on a smear of urinary deposit

Female: All kinds of organisms are found in the smears particularly

- ▶ Gram positive bacilli
- ▶ Gram negative cocci (Commensals)
- ▶ Culture is therefore essential

Culture is therefore essential (Fig.24)



Figure 24. Culture for Gonococci

DISPATCH OF SPECIMENS FOR CULTURE-

Using Martin & Laser's "Transgrow" medium

This is the best method, if the medium can be obtained from a specialized laboratory.

30-ml bottles contain 8 ml of solid medium (along one side of the bottle) are filled with a mixture of air (90%) and carbon dioxide (10%). The bottle should remain open for as short a time as possible to prevent the escape of gas

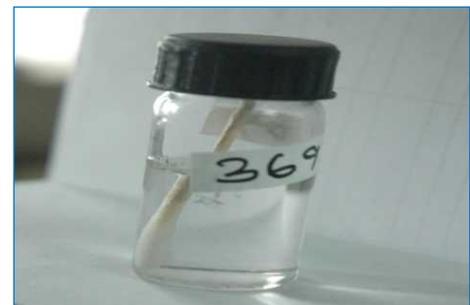


Figure 25 Gonococcal swab collection

1. Place the bottle upright. Collect the pus specimen on a swab. Unscrew the bottle cap.
2. Holding the bottle as upright as possible (to prevent the gas escaping), rub the swab of pus over the whole surface of the solid medium, from one side of the bottle to the other, from the bottom. (Fig.26)
3. Replace the cap on the bottle at once.
4. Dispatch at normal temperature.

Preservation time: up to 3 days, but the shorter the delay, the better.

This transport medium is also suitable for meningococci.

Using semi-solid transport medium for gonococci

Stuart transport medium can be used in small 5-ml bottles with stoppers.

1. Collect the pus specimen on a sterile cotton wool swab held in sterile (flamed) forceps.
2. Put the swab into the transport medium in the bottle.
3. Cut off the protruding portion of the swab stick with sterile (flamed) scissors.
4. Screw the cap on the bottle at once.

Preservation time: 6 hours only at normal temperature.

Using a Pasteur pipette

1. Draw the pus specimen into a sterile Pasteur pipette plugged with cotton wool.
2. Place the pipette as it is in a sterile tube, padded and plugged with cotton wool.

Preservation time: 6 hours only at normal temperature.

There are other media; in each case observe the instructions given by the specialized laboratory.

SYPHILIS

Syphilis is another important venereal disease, caused by a spirochaete: the pale treponeme (*Treponema pallidum*).

The first sign of the disease appears, usually on the genital organs as a chancre, round or oval, 1 or 2 cm in diameter, red, with firm, indurated edges.

Non-venereal (endemic) syphilis

This form of the disease is found in semi-desert regions such as the Sahel belt south of the Sahara and the Eastern Mediterranean area. It mainly affects children.

YAWS

This is a non-venereal disease found in humid tropical climates.

It is caused by a different treponeme (*T. pertenue*), which looks identical to *T. pallidum*.

Direct wet examination for treponemes in syphilis and yaws-

This can be carried out only by experienced personnel in a laboratory equipped with a dark field condenser microscope (Dark field microscope).

The examination is of no value when the patient has treated the lesion with ointment. In that case, wait 3 days before making the examination.

1. Clean the chancre with gauze moistened with sterile sodium chloride solution. Dry it.
2. Scrape the edges of the chancre several times with the flat blade of a sterile lancet. Do not draw blood.
3. Press with dry sterile gauze.
4. Remove the swab and wait a few minutes until a pinkish serous fluid appears.
Draw off the fluid with a Pasteur pipette with a teat.
5. Place a drop of the fluid on a thin glass slide (designed especially for dark ground microscopy).
6. Examine under the microscope using a dark field condenser.

The treponemes of syphilis and yaws can be distinguished from saprophytic treponemes of the skin by their very thin bodies and characteristic movement.

The technician needs special training to recognize them.

Examination of dried and stained smears- This is not recommended because of the presence of saprophytic treponemes on the skin and in the mucous membranes.

Serological examination (Fig.27) for syphilis and yaws, see VDRL test. This test should be repeated after 3-4 weeks if the examination for treponemes is negative.



Figure 27 Serology for syphilis

ANNEXURE - VI

URINE EXAMINATION

Urinary Deposits

Principle

Urine contains microscopic elements in suspension (cells, crystals, etc). These elements are collected by centrifuging and a drop of the deposit is examined between a slide and coverslip. As all these elements in suspension would sediment in the urine if left for a few hours, they are called urinary deposits.

Collection of urine

Examine a specimen passed in a single urination.

Examine a **mid-stream** specimen of fresh urine as soon as possible; it should be

- ▶ Collected in the laboratory
- ▶ Or brought quickly from the patient's room (within 2 hours of voiding)
- ▶ The sterile receptacle should be provided by the laboratory
- ▶ Women should be instructed to wash the genitalia before hand
- ▶ Men should collect urine sample after cleaning and retracting the prepuce
- ▶ Never carry out the examination on urine kept in the refrigerator

Value

In certain diseases of the urinary tract the urinary deposits are considerably altered. The following elements may be found:

- ▶ Pus cells
- ▶ An abnormal number of red blood cells (RBC)
- ▶ Parasitic forms
- ▶ Casts

Preservation of urine – can be done with formaldehyde solution, by adding 8 drops of 10% formaldehyde solution per 300ml of urine.

Note - Urine treated in this way cannot be used for any other laboratory test.

Materials

- ▶ Table top centrifuge
- ▶ 15ml conical centrifuge tube
- ▶ Capillary dropping pipette (Pasteur pipette), if possible calibrated, to deliver 50 drops per ml
- ▶ Slide and coverslip, 20 X20 mm
- ▶ If necessary, 10% formaldehyde solution

Preparation of the deposit

1. Mix the urine gently
2. Pour immediately into a centrifuge tube until it is 3/4 full
3. Centrifuge at medium speed for 5 minute
4. Pour off the supernatant urine by inverting the tube quickly without shaking
(The supernatant urine can be used for biochemical tests)
5. Shake the tube to resuspend the deposit
6. Draw a few drops of the deposit into a pipette
7. Place 1 drop on a slide and cover with a coverslip
8. Number the slide with the number of the specimen
9. Examine under the microscope at once:
 - ▶ First using the x 10 objective
 - ▶ Then using x 40 objective
 - ▶ Without a color filter
 - ▶ With the condensers lowered enough (or the condenser aperture reduced) to make transparent elements visible

The following may be found in urine deposits

- ▶ Red blood cells (RBC)
- ▶ Leukocytes(WBC)
- ▶ Yeast cells
- ▶ *Trichomonas vaginalis*
- ▶ Spermatozoa
- ▶ Epithelial cell
- ▶ Casts
- ▶ Parasitic egg and larvae
- ▶ Crystals

A. Red Blood Cells

They may be:

- a) Intact: small yellowish discs, darker at the edges (8 μ m)
- b) Crenated: spiky edges, reduced diameter (5-6 μ m)
- c) Swollen: thin circles, increased diameter (9-10 μ m)

Note: There are normally no red cells in the urine.

Red cells can be found in the urine of women if the specimen has been taken during the menstrual period.

B. Leukocytes (white cells)

They may be:

- a) Intact: clear granular discs, 10-15 μ m (the nuclei may be visible)

- b) Degenerate: distorted shape, shrunken, less granular
- c) Pus: clumps of numerous degenerate cells

The presence of many leukocytes, especially if in clumps, usually indicates a urinary tract infection.

How to express the quantity of red and white cells found in urine deposits-

It is important to mention the quantity of the various elements found. It is important always to use the same method of expressing quantities found.

With

1 drop of urine deposit (1/50 ml)

1 coverslip, 20X20 mm

X 40 objective; eyepiece x5 or x6

Examine microscopically

Red Cells

0-10 red cells per field - few red cells (normal)

10-30 red cells per field - moderate number of red cells

Over 30 red cells per field - many red cells

Leukocytes

0-10 leukocytes per field - few leukocytes (normal)

10-20 leukocytes per field - moderate number of WBC

20-30 leukocytes per field - many leukocytes

Clumps of more than 20 degenerate leukocytes - many leukocytes seen in clumps

Clumps and many degenerate Leukocytes - full field

C. Yeasts

Do not confuse with red cells

Size - 5-12µm

Shape - round or oval bodies of various sizes found together
Budding may be seen

They are not soluble in acetic acid.

Yeasts are occasionally present in urine containing glucose. Check that the urine is fresh.

D. *Trichomonas vaginalis*

Size - 15µm

Shape - round, globular

Motility - motile in fresh urine (they whirl and turn)

Undulating membrane - on one side

Flagella - 4 flagella, more or less visible

E. Spermatozoon

Occasionally found in the urine of males.

Head	-	very small (5µm)
Flagellum	-	long and flexible (50µm)
Motility	-	motile in very fresh urine

F. Epithelial cells

1. Squamous epithelial cells- Large rectangular cells, the product of desquamation (the shedding of cells from the epithelium of the urinary tract and organs). They come from:
 - The ureter or
 - The vagina
2. Bladder cells- Large cells often diamond shaped with a distinct nucleus
3. Cells from pelvis of the kidney- Medium sized cells (the size of 3 leukocytes) granular, with a sort of tail
4. Cells from the ureter and pelvis of the kidney. Medium sized oval cells with a distinct nucleus. If many are present together with leukocytes and filaments, they may be from the ureter. If few present, with no leukocytes, they may be pelvic cells.
5. Renal cells- Renal cells are small. They are the size of 1-2 leukocytes, very granular. The nucleus is refractile and clearly visible. They are almost always present with protein in the urine.

G. Casts: Casts are cylindrical in shape and long, crossing almost the whole field when examined under the 40x objective. They are formed during disease in the renal tubules, which may fill with blood and other cells and chemical deposits.

1. Hyaline casts: Transparent and slightly retractile, the ends rounded or tapered. (They may be found in healthy persons after strenuous muscular effort).
2. Granular Casts: Rather short casts filled with large granules pale yellow in color, with rounded ends (The granules come from degenerate epithelial cells from the tubules of the kidney).
3. Fine granular casts: The granules are smaller and do not fill the cast. Do not confuse with hyaline casts partly covered by amorphous phosphate crystals.
4. Blood Casts- Casts filled with more or less degenerate red blood cells, brownish in color.
5. Pus Casts: Casts filled with degenerate leukocytes. True pus casts are completely filled with leukocytes. Hyaline casts may contain a few leukocytes.
6. Epithelial casts : Casts filled with pale yellow epithelial cells (to make the cells more distinct, add a drop of 100 g/l (10%) acetic acid to the deposit)
7. Fatty casts(rare) : Very refractile yellowish casts, the edges indented and distinct, the ends rounded. Fatty casts are soluble in ether but not in acetic acid. (They are found in cases of severe kidney disease).

8. False casts : Do not mistake for casts:
 - Masses of phosphate crystals, short and clear-cut
 - Masses of translucent mucus, the ends tapering into threads
9. Miscellaneous foreign substances :If dirty receptacles or slides are used or if the urine specimen is left exposed to the air, the following may be found:
 - a) Oil droplets (refractile)
 - b) Starch granules (blue black with Lugol iodine solution)
 - c) Grains of pollen from flowers
 - d) Hairs
 - e) Cotton fibres
 - f) Air bubbles

H. Eggs and larvae of parasites

1. Eggs of *Schistosoma haematobium*: found together with red cells
2. Microfilaria of *W. bancrofti*: the urine appears white and cloudy

I. Crystals:

Crystals have regular geometric shapes unlike amorphous debris, which is made up of clumps of small granules with no definite shape.

A. NORMAL CRYSTALLINE DEPOSITS

1. Calcium oxalate (acid urine)
 - a) Shape like an envelope
Size 10-20µm[1-2 red cells]
 - b) Shape like a whole peanut
Size about 50µm very refractile
2. Uric acid (acid urine)
 - Shape various (square, diamond-shaped, cubical or rose-shaped)
 - Size 30-150µm
 - Color yellow or brownish-red
3. Triple phosphates (neutral or alkaline urine)
 - Shape rectangular or like a fern leaf or star
 - Size 30-50µm
 - Color colorless, refractile
4. Urates (alkaline urine)
 - Shape like a cactus or a bundle of needles
 - Size about 20 µm (2-3 red cells)
 - Color yellow refractile
 - (often found together with phosphates)

5. Less common crystals
 - A. Calcium phosphate (neutral or alkaline urine)

Shape	star-shaped
Size	30-40 μ m
Color	none
 - B. Calcium carbonate (neutral or alkaline urine)

Crystals- very small like millet or corn grains, grouped in pairs

Color	none
-------	------

(if 100 g/l (10%) acetic acid is added they dissolve, giving off bubbles of gas)
 - C. Calcium sulfate (acid urine)

Shape	long prisms or flat blades separate or in bundles
Size	50-100 μ m

(they can be distinguished from calcium phosphate crystals by measuring the pH of the urine)

B. AMORPHOUS DEBRIS

1. Amorphous phosphates (alkaline urine)

Granules small, whitish, often scattered

They are soluble in 100 g/l acetic acid (1 drop per drop of deposit)
2. Amorphous urates (acid urine) : Granules very small, yellowish, grouped in compact clusters. They are not soluble in 100 g/l acetic acid but dissolve if the urine is gently heated. [Urine kept in the refrigerator often shows a heavy precipitate of urates]

C. OTHER CRYSTALLINE DEPOSITS

The following are rarely found in the urine. When present, however, they are found in large quantities.

1. Cystine (acid urine)

Shape	hexagonal plates
Size	30-60 μ m
Color	colorless, very refractile

Found only in fresh urine, as they are soluble in ammonia.
[Found in cystinuria, a hereditary disease.]
2. Cholesterol [acid urine]

Shape	squarish plates, with notches on one side
Size	50-100 μ m
Color	colorless refractile

Soluble in ether
3. Bilirubin (very rare)

Shape	various tiny crystals, square or like beads or needles
Size	5 μ m (about 1/2 red cell)

Color brown

(the chemical test for bile pigments is positive)

4. Acetyl sulfonamides (neutral or acid urine)

Found in patients following treatment with Sulfonamide, crystals are varied in shape but most frequently like sheaves of needles. If large quantities of unidentified crystals are seen find out whether the patient is on sulfonamide therapy. The presence of these crystals should be reported because they can cause kidney damage.

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