CHAPTER 10

# LABORATORY DIAGNOSIS OF COMMON REPRODUCTIVE TRACT AND SEXUALLY TRANSMITTED INFECTIONS

**Abstract**

Laboratory tests are essential for confirming the clinical diagnosis in symptomatic individuals and diagnosing infection in asymptomatic individuals. Vaginal discharge and urethral and endocervical infection may be caused by a variety of pathogens. Syphilis is diagnosed by testing a patient's serum for antibodies to *Treponema pallidum*. The *non-specific* tests for syphilis include VDRL, RPR, and Wassermann tests. Specific serological tests detect the *specific* treponemal antibodies in the serum.

**Key Words**

Bacterial vaginosis, Reproductive tract infections, Sexually transmitted infections, Syphilis, *Trichomonas vaginalis*.

## 10.1 – BACTERIAL VAGINOSIS

Bacterial vaginosis (BV) was formerly called "non-specific vaginitis" or "anaerobic vaginitis". The new term implies that there is no infection, but a change in microbial flora of the vagina. BV is caused by an imbalance in the microbial flora of the vagina with overgrowth of anaerobic bacteria and lack of normal lactobacilli. This condition may be associated with presence of several pathogenic and non-pathogenic organisms, including anerobic bacteria (Goyal *et al*., 2004; Beigi *et al*., 2005; Hillier *et al*., 1993). The microbial flora of the vagina contains many anaerobic and facultative bacteria such as *Trichomonas vaginalis, Gardnerella vaginalis, Mobiluncus curtissi, Mobiluncus mulieris, Candida* spp., *Bacteroides, Peptostreptococcus, Provabella, Mycoplasma hominis,* and *Ureaplasma urealyticum*.

The estimated prevalence of this condition varies from 15 to 40 per cent in different populations. Indian studies have reported prevalence ranging from 11 to 71 per cent in women with abnormal vaginal discharge. Postulated *risk factors* include age at first sexual intercourse (Amsel *et al*., 1983), multiple sexual partners, use of vaginal medications, intrauterine contraceptive devices, and use of tobacco (Joesosef *et al*., 2001). Though sexual association has been

reported, the condition is also seen in lesbians (Marrazzo *et al*., 2002) and in women who have never been sexually active. The aetiopathogenesis of BV is still unclear but the following hypotheses have been put forward: (a) increased oestrogen concentration during the follicular phase of menstrual cycle favours growth of various pathogenic and non-pathogenic bacteria; (b) altered estrogen-progesterone ratio in pregnancy reduces prevalence of BV; (c) increase in mucinase and sialidase enzymes in vaginal discharge favours penetration of microorganisms; (d) endocrine changes causing disappearance of endogenous flora, facilitating growth of other bacteria; and (e) sexual transmission. In 50 per cent of women, BV is asymptomatic. The commonest symptom is whitish grey thin vaginal discharge, which has a characteristic fishy odour. The discharge is adherent to the vaginal wall. Other manifestations include vaginal pruritus and vulvovaginitis.

Diagnosis: New diagnostic tests (affirm VP III DNA probe test, QuickVue advance pH and amine test, QuickVue advance *G. vaginalis* test) have questionable sensitivity and hence are not widely used. According to the *composite clinical criteria* described by Amsel *et al*. BV is present if three out of the following four signs are present: (a) thin, homogeneous vaginal discharge; (b) vaginal pH more than 4.5; (c) positive amine odour with 10 per cent potassium hydroxide ("whiff test"); and (d) presence of "clue cells". Clue cells are denuded vaginal epithelial cells covered by bacteria in which the margins of these cells are not defined. The absence of clue cells indicates absence of BV, irrespective of vaginal pH, or clinical appearance of vaginal discharge. Thus, presence of clue cells is pathognomonic of BV. BV is defined as "cured" if three out of four criteria are absent and as "partially cured" if only two criteria are present (Amsel *et al*., 1983).

Grading of Gram-Stained Slides: In 1991, Nugent *et al*. described a cheap, simple, and objective scoring method for grading Gram-stained slides. Score for the following is summated to obtain a total score between 1 and 10 – large Gram-positive rods (*Lactobacillus* spp.), small Gram-positive rods (*Bacteroides* spp.), small Gram-variable rods (*G. vaginalis*), curved Gram-variable rods (*Mobiluncus* spp.), and Gram-positive cocci. A total score of more than 7 is diagnostic for BV (0–3 = normal vaginal flora; 4–6 = intermediate).

Treatment and Prognosis: Metronidazole and clindamycin are recommended for the treatment of BV (McDoland *et al*., 2005). The same treatment regimen is recommended for pregnant women also. The mutagenicity associated with long-term use of metronidazole in animals has not been reported in humans in the first trimester of pregnancy (Burtin *et al*., 1995; Piper *et al*., 1993). Both metronidazole and clindamycin are secreted in breast milk. In the recommended doses, these drugs are not harmful to the infant. Metronidazole acts on anaerobes, spares $H_2O_2$-producing lactobacilli and does not alter the normal vaginal flora. The recommended dose is 400 mg orally, twice daily, for 7 days. Metronidazole cream (0.75 per cent; 5 g *per vaginum* at night) is not recommended since it does not

reach the upper genital tract and is very expensive. However, metronidazole cream (1 per cent) may be used *per rectum* as a prophylactic prior to gynecological surgeries. Clindamycin is administered orally (300 mg twice daily for 7 days) or clindamycin cream (2 per cent; 5 g *per vaginum* at night). However, clindamycin destroys the $H_2O_2$-producing lactobacilli. Though BV is also sexually transmitted, studies on current treatment of the male partner have been inconclusive. In some women, BV may recur with the onset of menstruation and disappear during mid-cycle or it may follow vaginal candida infection. Avoiding vaginal douches and alkaline agents (soap, shower gels) in the genital region can prevent recurrence. Once-a-month treatment has been advocated for recurrent BV, but its efficacy is not proved.

## 10.2 – INFECTIONS IN NON-PREGNANT WOMEN

The acidic environment of the vagina and hydrogen peroxide produced by lactobacilli protect against many bacterial and viral infections. *G. vaginalis* reportedly acts as an HIV-1 inducing factor (Cohn *et al*., 2005). Antibodies to *Neisseria gonorrhoeae, Chlamydia trachomatis,* and *M. hominis* have been demonstrated in serum from patients with acute salpingitis (Mardh *et al*., 1981) while antibodies to *M. hominis* have been demonstrated in serum from patients with genital infections (Mardh & Westrom, 1970b).

Bacterial Vaginosis: The production of CD4 lymphocytes is blocked; while that of interleukin-10 is increased. It has been found to be associated with adnexal tenderness, indicative of pelvic inflammatory disease (Eschenbach *et al*., 1988). It has also been found to act as a cofactor to HPV in causing carcinoma in situ (Platz-Christensen *et al*., 1994). Pre- and post-operative metronidazole therapy has been shown to reduce the frequency of vaginal cuff infection from 9.5 to 2 per cent in women with BV who underwent abdominal hysterectomy (Larsson & Carlsson, 2002). *M. hominis*, an organism associated with BV, has been isolated from tubal and cervical cultures (Mardh & Westrom, 1970a). There is an increased risk of post-abortal upper genital tract infection in women who had "clue cells" in their vaginal secretions. Initiation of antibiotic therapy before medical termination of pregnancy (MTP) was found to have a protective effect (Crowley *et al*., 2001).

## 10.3 – INFECTIONS IN PREGNANT WOMEN

Pregnant women with BV have a 40 per cent higher risk of preterm birth. Preterm birth accounts for 8–10 per cent of all births and is a major cause of neonatal morbidity and mortality. In patients with BV, peptostreptococci, and *G. vaginalis* are the common causes of endometritis that may occur within 2 days after delivery by caesarean section. This is because infection is introduced directly into the endometrium. However, in case of normal vaginal delivery, endometritis may occur up to 6 weeks after delivery due to ascending infection. In view of the documented adverse pregnancy outcomes, asymptomatic pregnant

women with a past history of BV should be ideally screened for this condition (Coli *et al*., 1996; Silver *et al*., 1989). Earlier the infection in pregnancy, higher is the risk of preterm birth. Treatment of BV in pregnant women reduced the risk by 27–50 per cent (Coli *et al*., 1996). Amniotic fluid from patients with chorioamnionitis has been found to contain *G. vaginalis*, *M. hominis*, and some anerobes (Silver *et al*., 1989).

## 10.4 – NEED FOR LABORATORY DIAGNOSIS

Community-based studies reveal that common reproductive tract infections (RTIs) in women are cervicitis, pelvic inflammatory disease, and vaginitis (Aggarwal & Kumar, 1999; Bang *et al*., 1989). Though preventable and treatable, inadequate treatment or neglect of RTIs may lead to serious complications like infertility, ectopic pregnancy, pregnancy wastage, and low birth weight (Misra *et al*., 1997).

The frequency of vaginal discharge as reported in several studies (Thakur *et al*., 2002; Palai *et al*., 1994; Das *et al*., 1994; Kambo *et al*., 2003; Nandan *et al*., 2001) varies from 4.9 per cent (Kambo *et al*., 2003) to 71.17 per cent (Ram *et al*., 2006). A community-based study (Aggarwal & Kumar, 1999) in rural Haryana (on ever-married women of reproductive age), and a syndromic approach-based study (Ranjan *et al*., 2003) on married women have reported RTIs in 70 per cent and 37 per cent of respondents, respectively.

Most RTIs go unrecognised and are considered as "normal" by women. In HIV-positive individuals, the clinical presentation and course of STIs may be modified. In such cases, laboratory diagnosis is essential. Due to the limitations of syndromic management (Gupta & Mahajan, 2003) laboratory tests are essential for confirming clinical diagnosis and diagnosing infection in asymptomatic individuals.

## 10.5 – TESTS FOR VAGINAL DISCHARGE

Vaginal discharge, a common complaint, may be due to infection of vagina, cervix, and/or uterus. The possible pathogens include *T. vaginalis, G. vaginalis, M. curtissi, Bacteroides* spp., *M. mulieris*, *Candida* spp. and *Peptostreptococcus*.

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscope slides, spirit lamp or bunsen burner, vaginal speculum, freshly prepared reagents, and sterile physiological saline.

Collecting Vaginal Specimen: (a) moisten the speculum with sterile warm water and insert it into the vagina (do not lubricate the speculum with a gel that may be bactericidal); (b) cleanse the cervix using a swab moistened with sterile physiological saline; (c) pass a sterile cotton wool swab for about 20–30 mm into the endocervical canal and gently rotate the swab against the endocervical wall, to obtain a specimen; and (c) when gonorrhoea is suspected, inoculate the Petri dish containing the culture medium, taking all sterile precautions.

Examination and Interpretation:

### 10.5.1 – Colour and Odour

1. Yellow-green purulent discharge (*T. vaginalis*)
2. White colourless discharge (*Candida albicans*)
3. Grey, offensive smelling (fishy ammoniacal), thin discharge (*G. vaginalis*)

### 10.5.2 – pH Test

The normal pH of the vaginal discharge from puberty to menopause is *acidic* and the pH ranges from 3.0 to 3.5. The pH can be measured using Whatman pH paper.

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscopic slides, sterile physiological saline, spirit lamp or bunsen burner, vaginal speculum, Whatman pH paper.

Steps: Take pH indicator paper strips in the range of ±3.8 to ±6.0. Touch the specimen swab on the pH paper (or) 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 the vagina directly.

Precaution: The pH paper should not come in contact with the cervical secretions.

Interpretation: Normal adult vagina has a pH of 3.0–3.5. pH of more than 5.0 is seen in *Trichomoniasis* and BV; pH of less than 5 may indicate candida infection.

False Positive/Negative Results: Presence of menstrual blood, cervical mucus, and semen may also raise vaginal pH.

### 10.5.3 – Potassium Hydroxide Wet Mount

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscopic slides, sterile physiological saline, spirit lamp or bunsen burner, vaginal speculum, freshly prepared reagents.

Steps: (1) Place the vaginal discharge specimen on a clean grease-free microscope slide; (2) add two drops of 10 per cent potassium hydroxide (KOH, 10 g per 100 mL) on the specimen and mix well. KOH being corrosive, should be handled with care; (3) put a clean cover slip over the specimen, ensuring that there is no trapping of air bubble between the specimen and the cover slip; (4) pass the slide gently over a flame of the bunsen burner or spirit lamp for 10–20 seconds, taking care that the specimen does not boil; and (5) observe the slide under high power objective (×40) of light microscope.

Interpretation: Round or oval yeast cells of the size of 5–7 μm in diameter and the presence of mycelia or pseudohyphae are diagnostic of candidiasis (Cheesbrough, 2000).

Significance of this Test: A majority of cases of candidiasis can be diagnosed by this method. Saline wet mount can also be used as a substitute for KOH, but adding KOH is better since the mycelia are separated and clearly visible because it digests other epithelial cells and debris.

### 10.5.4 – Wet Mount

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscopic slides, sterile physiological saline, spirit lamp or Bunsen burner, vaginal speculum, freshly prepared reagents.

Steps: (1) use a sterile swab to collect a specimen from the vagina; (2) transfer a sample of the exudate on a clean grease-free microscope slide. Alternatively, a drop of vaginal fluid can be used, if available; (3) add a drop of *sterile* physiological saline and mix; (4) cover with a cover slip; (5) observe the slide first under ×10 magnification. Any field, which shows the suspected organism, or parasite, is seen under ×40 magnification of light microscope.

Precautions: The preparation should not be too thick and should be examined as soon as possible, after the specimen is collected. Only *sterile* saline or saline that is checked daily by the laboratory should be used to exclude contamination by motile organisms that can be mistaken for *T. vaginalis*. In tropical climates, saline solutions get easily contaminated. The condenser and iris diaphragm should be sufficiently closed to give good contrast.

Interpretation: Trophozoites of *T. vaginalis* are larger than pus cells, measuring 10–20 µm in diameter. They are round or oval in shape. There are four anterior flagella, and a fifth flagellum forms an undulating membrane. The parasite moves actively with jerky movements. Incubating the preparation in a Petri dish containing a damp piece of cotton wool, at 35–37°C for a few minutes can revive motility. The presence of "clue cells" suggests the diagnosis of BV. These cells do not have a well-defined edge because of the presence of bacteria and the disintegration of cells (Cheesbrough, 2000).

### 10.5.5 – Amine Test

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscopic slides, sterile physiological saline, spirit lamp or Bunsen burner, vaginal speculum, freshly prepared reagents.

Steps: (1) take a drop of vaginal fluid on a clean, grease-free microscope slide; (2) add one drop of 10 per cent KOH on the vaginal fluid; and (3) bring the slide close to the nose and smell immediately.

Precautions: The preparation becomes odourless soon after the test is performed; hence should be read immediately.

Interpretation: An intense putrid fishy odour indicates a positive reaction, which is suggestive of infection with BV organisms such as *G. vaginalis*, or with anaerobes such as *Bacteroides*, *Peptostreptococcus*, and *Mobiluncus*.

### 10.5.6 – Hydrogen Peroxide Test

This test is very simple and can be easily performed at peripheral laboratories (Cheesbrough, 2000).

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscopic slides, sterile physiological saline, spirit lamp or Bunsen burner, vaginal speculum, freshly prepared reagents.

Steps: (1) take a drop of vaginal fluid on a clean grease-free slide; (2) add a drop of 3 per cent hydrogen peroxide on the specimen; and (3) on mixing, "foaming bubbles" (effervescence) is seen on the slide.

Precautions: If the specimen is collected from the endocervix (in a case of gonorrhoea, or chlamydia infection), a *false positive* test for *trichomoniasis* may be obtained.

Interpretation: presence of "foaming bubbles" (effervescence) indicates the presence of white blood cells, which are seen in *trichomoniasis*.

## 10.6 – TESTS FOR URETHRAL AND ENDOCERVICAL DISCHARGE

Possible pathogens are *Neisseria gonorrhoea, Streptococcus pyogenes, U. urealyticum, C. trachomatis*, and *T. vaginalis* (occasionally). It is essential to collect the specimen from the site of the lesion, without touching the surrounding area, for the correct laboratory confirmation of clinical diagnosis.

Materials Required: sterile gloves, sterile cotton wool swab sticks, grease-free microscopic slides, sterile physiological saline, spirit lamp or Bunsen burner, vaginal speculum, freshly prepared reagents, and glass marking pen.

Steps for Collecting Urethral Discharge: Collect the specimen while wearing sterile gloves. Cleanse around the urethral opening using a swab moistened with sterile physiological saline. Gently massage the urethra from above downward and collect the pus with a sterile cotton wool swab. If there is no discharge, insert a sterile thin cotton wool swab 2–3 cm into the urethra and rotate for 5–10 seconds to scrape the mucosa. Put the swab in Amie's transport medium or sterile test tube and label (Cheesbrough, 2000).

Steps for Collecting Endocervical Discharge: Moisten the vaginal speculum with sterile warm water and insert the speculum into the vagina. Do not lubricate the speculum with a gel that may be bactericidal. Cleanse the cervix with a swab moistened with sterile physiological saline. Inspect the exocervix for lesions and

insert another cotton wool swab up to 2 cm into the cervical canal. Rotate the swab for 5–10 seconds and withdraw. Put the swab in Amie's transport medium, or sterile test tube and label. (Cheesbrough, 2000).

Precautions: The patient should not have passed urine, preferably for 1–2 hours before the specimen is collected. Antiseptics should never be used, as very delicate organisms like the *gonococci* are likely to get destroyed. The specimen should be processed *immediately* in the laboratory, as the organisms are highly autolytic and may not be visible if there is a delay in processing the sample.

Microscopic Examination: Take a microscopic slide and place a drop of water on it. If the slide is clean and grease-free, a thin film of this drop can be made on the slide. Otherwise, water collects on the slide in the form of fine droplets and a film can not be made.

### 10.6.1 – Preparing the Smear

1. Take a clean *grease-free* microscopic slide and wipe it with gauze. The slide should be free from scratches. Pass the slide 2–3 times through the flame of a Bunsen burner or a spirit lamp in order to remove traces of grease from the slide.
2. Mark the central part of the slide with two vertical lines, 2–3 cm apart, with the help of a glass-marking pen.
3. Roll the cotton wool swab with the specimen, on to this marked area on the slide. *Note*: When making a smear, a swab of the discharge should be gently rolled on the slide to avoid damaging the pus cells. This helps in better visualisation of intra-cellular *gonococci*.
4. Make a smear of the size of $2 \times 1$ cm. Allow the smear to dry in air. *Note*: Heat fixation is contraindicated if gonococcal infection is suspected.
5. Label the smear on the right or left-hand corner of the slide.

### 10.6.2 – Heat Fixing the Smear

The objective of heat fixing is to: (a) prevent autolytic changes in the smear and preserve the smear and to prevent the wash out of the smear during the staining process; (b) make the organisms more permeable to different stains, thus giving good staining characters; and (c) render the smear non-infectious to some extent.

Steps for Heat Fixing: Hold the slide in such a way that the smear is on the upper side. Pass the slide over the flame of a Bunsen burner or a spirit lamp twice or thrice. Now judge the temperature of the slide by feeling it on the back (dorsum) of the hand. The slide should be hot enough for the heat to be intolerant.

Precautions during Heat Fixing: *Excess heating* will char the smear and nothing can be visualized under the microscope. On the other hand, with *inadequate heating*, the smear may get washed out during the staining process and nothing can be visualized under the microscope.

Contraindication for Heat Fixation: Since the *gonococci* are delicate organisms, heat fixation is contraindicated. Instead of heat fixing, methanol is used for fixing the smear.

### 10.6.3 – Reagents for Gram's Stain

- Crystal Violet – Solution A: crystal violet powder (20 g) in 200 mL ethyl alcohol. Solution B: 8 g ammonium oxalate in 800 mL distilled water. Mix Solutions A and B, and filter.
- Gram's Iodine – 20 g resublimated iodine is mixed in 100 mL one normal NaOH solution (4 g sodium hydroxide in 100 mL distilled water).
- Acetone-Alcohol – 100 mL acetone in 500 mL ethyl alcohol.
- Safranin – The stock solution contains 10 g safranin in 200 mL ethyl alcohol. The working solution is prepared by diluting 100 mL of the stock solution in 900 mL of distilled water.

### 10.6.4 – Procedure for Gram's Staining

1. Cover the smear with crystal violet solution (the "dye") and allow it to act for 20–30 seconds. *Note*: Flooding the whole slide will only cause wastage of reagents. Pour the crystal violet solution and wash the slide under slow running water, keeping the slide at an angle, so that only the crystal violet solution is washed out and not the smear.
2. Cover the smear with freshly prepared Gram's iodine solution and allow it to act for 30 seconds. This acts as a mordant, by forming a dye-iodo complex.
3. Wash the slide under slow running water and add acetone-alcohol mixture. This acts, as a decolorizing agent and the end point of decolourisation is that violet colour ceases to come off the slide. This can be confirmed by holding the slide against a white background. (Absolute alcohol, i.e. 100 per cent ethanol can be used as a substitute for acetone-alcohol mixture for decolourisation.) This is a critical stage of Gram's staining, as overdecolourisation can make a Gram-positive organism to appear as Gram negative, and lead to faulty diagnosis.
4. After washing the slide with water, pour safranin on the slide and allow it to act for 1–2 minutes. The purpose of safranin is to counterstain the Gram-negative organism, pus cells and the background.
5. Wash the slide with water and gently blot the slide dry, between two blotting papers. Put a drop of liquid paraffin on the stained smear and observe under oil immersion lens (×100).

### 10.6.5 – Microscopic Examination

1. Put a drop of liquid paraffin on the stained smear and observe under oil immersion lens (×100).

2. Push the condenser of the microscope upwards and open the iris diaphragm so that maximum light passes through the smear. This helps in a clear visualisation of the organisms, pus cells, and other elements.
3. Examine the smear for epithelial cells, polymorphonuclear leukocytes (pus cells), organisms and their location – whether extracellular or intracellular.

Interpretation: The ideal oil immersion field for microscopic examination is the one that which shows a clear contrast between Gram-positive material (stained *purple*) and Gram-negative material (stained *pink*). The severity of the infection can be judged from the number of organisms and pus cells per oil immersion field. *Gonococci* are Gram negative (stained *pink*), intracellular, bean-shaped, and are usually arranged in pairs ("diplococci"). In asymptomatic *gonococcal endocervicitis*, *gonococci* may not be visible under Gram's stain. Culture methods are required in such cases. If the whole slide appears *pink*; this indicates excessive decolourisation.

## 10.7 – LABORATORY TESTS FOR SYPHILIS

Syphilis is primarily transmitted through sexual contact, though it can also be spread by blood transfusion or by percutaneous route, as in occupational exposure. The disease has an *early infectious stage*, which occurs within the first 2 years of infection, this includes: (a) primary syphilis (a characteristic primary lesion called "chancre" appears at the portal of entry and heals spontaneously); (b) secondary syphilis (a generalised skin eruption appears after about 6 weeks later; and (c) early latent syphilis. The *late non-infectious stage* comprises: (a) late latent syphilis; (b) benign late syphilis; (c) cardiovascular syphilis; and (d) neurosyphilis. This stage occurs about a decade after the occurrence of the primary lesion. Degenerative and irreversible necrotic lesions that do not contain treponemes are characteristic of late non-infectious stage.

Direct demonstration of *T. pallidum* by dark-field microscope is the simplest and the most rapid method of diagnosing syphilis. Smears obtained from chancre or mucous patches are stained by Fontana's stain or by silver impregnation technique. *T. pallidum* can also be demonstrated in biopsies of tissues by silver impregnation or by immunofluorescence. However, the organisms can not be detected after the primary stage of syphilis, when the lesions have healed. Though PCR is available, it has not yet been standardised. Therefore, the main method for diagnosing syphilis is by testing a patient's serum for antibodies to *T. pallidum*.

### 10.7.1 – Collection of Blood Sample

Materials Required: sterile gloves, tourniquet, disposable syringe and needle no. 21 or 22 gauge, sterile cotton wool, discarding jar containing 1 per cent sodium hypochlorite solution, spirit, centrifuge, sterile dry test tubes, and Pasteur pipettes.

*10.7.1.1 – Steps for collecting blood sample*

1. Apply tourniquet above the cubital fossa.
2. Ask the patient to clench his or her fist, with the thumb facing inside, so that the veins become prominently visible.
3. Clean the region around the cubital fossa with spirit swab.
4. Wear gloves and feel the antecubital veins.
5. With the help of sterile disposable needle and syringe, collect 3–5 mL of venous blood.
6. Open the tourniquet, remove the needle from the vein and apply pressure on the site with spirit swab.
7. Transfer the collected blood in a properly labelled sterile test tube and allow it to clot for 1 hour.
8. Discard the syringe and needle in the discarding jar containing 1 per cent sodium hypochlorite solution.
9. Remove the supernatant serum into another sterile test tube with the help of Pasteur pipette.
10. Centrifuge the test tube at 1500 revolutions per minuted (RPM) for 15 minutes. Any red cells transferred to this test tube will settle down after centrifugation.
11. Remove the serum with a Pasteur pipette in to a sterile screw cap bottle.
12. Label the vial and store in the refrigerator at 4°C, till further use.
13. Store the vial in the freezer of the refrigerator, if needed in future.

*10.7.1.2 – Precautions during blood collection*

1. During blood collection, avoid needle-stick injuries.
2. Any breach in the integrity of the skin such as abrasion, wound, or injury should be covered with waterproof adhesive tape and sterile gloves should be worn.
3. Hot (autoclaved) sterile syringes may haemolyse the blood; and should not be used.
4. The test tube should be carefully labelled with the patient's name, age, sex, date of collection, and registration number.

### 10.7.2 – Serological Tests for Syphilis

Serological tests can be used for the diagnosis of late primary, secondary and late syphilis. Two major groups of antibodies are produced by the immune system of the host, who is infected by *T. pallidum*.

*10.7.2.1 – Non-specific antibody*

About 1–3 weeks after the appearance of the primary lesion, a substance called *reagin* (an antibody complex) appears in the serum. The presence of reagin is

detected by serological tests that use non-treponemal antigen i.e. antigens from beef heart. Two types of non-treponemal tests are used to detect and measure *reagin* in the serum.
(a) Flocculation Tests: VDRL test and rapid plasma reagin (RPR) card test.
(b) Complement Fixation Test: Wassermann reaction.
False positive results may be obtained with sera from healthy individuals or patients suffering from other diseases because these tests detect non-specific antigens that are shared by treponemes and mammalian tissues. "Acute" biological false positive results turn "negative" within 6 months in viral infections such as measles, infectious mononucleosis, mycoplasma pneumonia, and malaria. "Chronic" biological false positive results persist for 6 months or longer and may occur in leprosy and autoimmune diseases.

*10.7.2.2 – Specific treponemal antibody*

This reacts with antigens prepared from live or dead treponemes, or those prepared from extracts of virulent Nichol's strains of *T. pallidum* maintained in rabbit testicle. The treponemal tests include:
1. Fluorescent treponemal antibody absorption test (FTA-ABS).
2. Micro-haemagglutination assay for *T. pallidum* (MHA-TP).
3. *T. pallidum* immobilization (TPI) test.
4. Enzyme immunoassay (EIA).
5. *T. pallidum* haemagglutination (TPHA) test.
6. *T. pallidum* particle agglutination (TP-PA) test.

Venereal Disease Research Laboratory (VDRL) and TPHA are used as *screening tests* in pregnant women, blood donors, and "at risk" patients. VDRL is more sensitive than TPHA in early syphilis, while the converse is true in latent and late stages of the disease. FTA-ABS and TPPA are *confirmatory tests*, to be used when one screening test is positive.

*10.7.2.3 – VDRL test*

The acronym VDRL stands for "Venereal Disease Research Laboratory". In this test, *reagin* antibodies in patient's serum are detected by "cardiolipin antigen" (an alcoholic extract of bovine heart muscle, to which lecithin and cholesterol are added).

Materials and Equipment Required: round bottle (30 mL capacity), water bath, micropipettes to deliver 20–60 µL, VDRL slide with depressions (14 mm in diameter), antigen (0.5 mL vial), buffered saline, and mechanical rotator.

Preparation of Fresh Antigen: Transfer 0.4 mL of buffered saline to a 30 mL round bottle by micropipette. Add 0.5 mL of the antigen (available commercially) directly to the saline while gently rotating the bottle on a flat surface. Add antigen over a period of 6 seconds and continue rotating for 10 seconds. Add 4.1 mL of buffered saline and mix well by repeatedly inverting the bottle about 30 times in 10 seconds.

Procedure for Qualitative VDRL Test: Inactivate the patient's serum by heating in a water bath at 56°C for 30 minutes. Add 60 µL of inactivated serum in VDRL slide. Add 20 µL of fresh antigen to the inactivated serum in VDRL slide. Mix and rotate the slide for 4 minutes in a mechanical rotator (to be set at 180 RPM). Read the test microscopically. Report the results as: (a) "reactive"; (b) "weakly reactive"; or (c) "non-reactive".

Procedure for Quantitative VDRL Test: Dilute the serum in geometric progression and titrate these dilutions with freshly prepared antigen. The quantitative result is reported as the highest dilution in which the test is fully reactive. VDRL titre is between 1:8 and 1:16 in primary syphilis, and between 1:16 and 1:128 in secondary stage, cardiovascular and neurosyphilis.

*10.7.2.4 – RPR card test*

The acronym RPR stands for *rapid plasma reagin*. The RPR card test is commercially available as a ready-to-use kit. The test is performed according to the manufacturer's instructions supplied with the kit. In this test, finely divided carbon (charcoal) particles and choline chloride are added to cardiolipin (i.e. VDRL antigen).

Advantages over VDRL Test

1. Simpler than the VDRL test, it can also be performed at the peripheral health centres (Cheesbrough, 2000).
2. Addition of finely divided carbon particles enables visual reading of results and the reactivity of the antigen is enhanced.
3. There is no need for heat inactivation of the sample.
4. Plasma as well as serum can be used in the RPR test and blood from finger prick is sufficient.
5. Can be tested on plastic or paper cards.

Its disadvantage is that it can not be used with CSF. RPR card test kit contains the following materials: (a) plastic-coated card with circles on them; (b) antigen suspension in an unbreakable container; (c) 20 gauge needle without bevel; and (d) disposable plastic stirrer.

Other materials needed, but not provided with the kit are: (a) normal (physiological) saline; (b) pipettes (automatic or glass) – 1 mL, 2 mL, and 0.5 mL (with 0.01 mL calibrations); and (c) mechanical rotator adjusted to rotate forming a circle, 2 cm in diameter. Speed should be adjusted to the number of RPM, as per kit specifications.

Steps: Take one test-card and with the help of a pipette, place 0.05 mL of the *unheated* serum on one of the circles. Spread the serum sample on the circle, using the disposable plastic stirrer. Gently shake the RPR card test antigen, and put one drop (1/60 mL) of this antigen in the serum sample of the circle. Rotate the card on the mechanical rotator, after adjusting the speed. The duration and the number of RPM should be as per the instructions supplied with the kit. Remove the card from the rotator, and see the results *immediately* with naked eye in bright light.

Results and Interpretation: Formation of small to large clumps indicates that the test is "reactive". Lack of clumps or presence of slight roughness indicates a "non-reactive" test.

Caution: False positive results may be obtained in conditions like viral pneumonia, malaria, leptospirosis, tuberculosis, and connective tissue disorders such as disseminated lupus erythematosus (Gupta & Mahajan, 2003).

*10.7.2.5 – Fluorescent treponemal antibody absorption test*

In the FTA-ABS test, the patient's serum is absorbed with an autoclaved supernate from cultures of treponemes in order to remove group-specific antibody. Binding of *T. pallidum*-specific antibody is demonstrated by indirect immunofluorescence method. This test can detect IgG and IgM antibodies. It is the earliest serological test that becomes positive and remains positive for many years, even after treatment.

*10.7.2.6 – Micro haemagglutination assay*

In the MHA-TP, tanned sheep erythrocytes are sensitised with extract of Nichol's strain of *T. pallidum* and then mixed with patient's serum. If the serum contains treponemal antibodies, the erythrocytes clump together.

*10.7.2.7 – Treponema pallidum IMMOBILIZATION (TPI)*

When serum of a syphilitic patient and complement are added to actively motile Nichol's strain of *T. pallidum*, these spirochetes are immobilized. Although this is the most specific test for diagnosing syphilis, it is difficult, time consuming, and expensive.

*10.7.2.8 – Enzyme immuno assay (EIA)*

This assay detects the antigen-antibody complex by using a tracer complex (that contains horse radish peroxidase conjugated monoclonal antibody to *T. pallidum*).

*10.7.2.9 – Treponema pallidum Haemagglutination (TPHA)*

Antibodies in patient's serum agglutinate sheep erythrocytes (coated with extract of *T. pallidum*). TPHA is often negative in early syphilis but may become positive at low titres (1:80 to 1:320) towards the end of the primary stage. The titre rises sharply during the secondary stage.

*Table 1.* Interpretation of serological tests for syphilis

| VDRL | TPHA | FTA-ABS | Interpretation |
|---|---|---|---|
| Positive | Negative | Negative | False positive reaction – repeat to exclude primary infection |
| Positive | –/+ | Positive | Primary infection |
| Positive | Positive | Positive | Untreated or recently treated |
| Negative | Positive | Positive | Fully or partially treated |
| Negative | Positive | Negative | Past history of treated syphilis |

*10.7.2.10 – Treponema pallidum Particle Agglutination (TP-PA)*

TP-PA is a specific serological test for the detection of antibodies in various species and subspecies of pathogenic treponemes, which cause syphilis, yaws, pinta, bejel, and endemic syphilis. This test is used to confirm the reactive results of a non-treponemal screening test for syphilis such as VDRL test. TP-PA is based on passive agglutination wherein serum samples containing antibodies to pathogenic treponemes react with gel particles (sensitised with sonicated antigens of Nichol's strain of *T. pallidum*) to form a smooth mat of agglutinated gel in the microfilter tray well. If antibodies are not present, the gel particles settle to the bottom of the tray well, forming a characteristic compact button of unagglutinated particles. The control well containing unsensitized gel should also show this compact button or absence of agglutination (Pope & Fears, 2000). SERUM SAMPLE: Collect *fresh* serum sample (about 0.5 to 1 mL, *never* less than 0.4 mL) using regular red top or serum separator vaccutainers and allow the specimen to clot at room temperature and centrifuge. Transfer to polypropylene screw-capped vial with capacity of 2 mL. Serum samples are stable up to 72 hours at 4°–8°C. For longer periods of storage, the recommended temperature is –20°C or lower. Repeated freezing and thawing may compromise the integrity of the specimen. Excessively haemolysed, contaminated or lipaemic sera may give atypical results and should not be used. A serum sample is considered too haemolysed to be tested if printed material can not be read through it. Heat-inactivated (56°C for 30 minutes) serum may be used. Excessive inactivation time or temperature may cause equivocal results.

### 10.7.3 – Interpretation of Serological Tests

Non-treponemal (non-specific) tests become negative or show decline in titres with effective therapy, while treponemal tests usually remain positive even after successful therapy.

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