

ANNEXURES

LABORATORY TESTS FOR RTIs/STIs

Laboratory tests improve the diagnostic sensitivity and specificity of symptomatic RTIs/STIs, particularly in women, to differentiate serious infections, i.e., cervicitis, from milder but more common infections, i.e., vaginitis. Simple laboratory tests incorporated in syndromic management of urethral discharge also help distinguish between mixed and single infections, reducing the administration of unnecessary antibiotics. The tests also help in detection of infections in asymptomatic individuals, specifically in female Clients, who carry the burden of RTIs/STIs complications and sequelae. Laboratory testing is even more important in pregnant women to prevent the adverse consequences of syphilis, gonococcal and chlamydial infection in newborns.

Laboratory diagnosis of RTIs includes three major equally important steps i.e; collection of specimen, its transport and use of a reasonable sensitive and specific test. Laboratory procedures at PHC level should include microscopic examination of fresh and stained specimens. Microscopic examination of urethral discharge helps to single out nongonococcal infection. Wet mount microscopy in vaginal discharge helps to detect trichomoniasis, (*Trichomonas vaginalis*) candidiasis and bacterial vaginosis (BV). Simple additional tests to identify bacterial vaginosis are the KOH sniff test and measurement of pH of vaginal fluid. Lab procedures may also include simple nontreponemal syphilis screening tests: rapid plasma reagin (RPR) or Venereal Disease Research Laboratory (VDRL).

Effective diagnosis of vaginitis by vaginal pH, amine test and wet smear of vaginal smear can be achieved with a sensitivity of 75-80%. The sensitivity of detecting candida organisms by 10% KOH preparation, saline microscopy and Gram stain is 70%, 40-60% and 65% respectively. The sensitivity of wet mount to identify trichomonads in symptomatic women is approximately 80% while it decreases to 50% in asymptomatic women. The sensitivity of papanicolaou (PAP) smear for *T. vaginalis* is around 60%. Gram stain is more reliable than PAP for diagnosis of BV infection. For other RTIs/STIs, it is advisable to use ELISA based assays or molecular diagnostics to achieve good sensitivity and specificity.

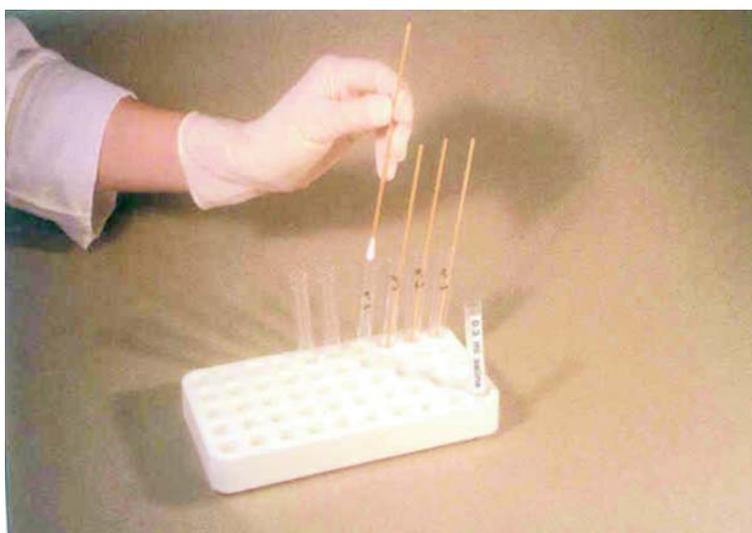


Fig A1a : Collection of specimen on swab

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VAGINAL PH

The pH of vaginal fluid should be measured using pH paper of appropriate range (3.8 to 6.0). The vaginal fluid sample is collected with a swab from the lateral and posterior fornices of the vagina and the swab is then touched directly on to the paper strip. Alternatively, the pH paper can be touched to the tip of the speculum after it has been withdrawn from the vagina. Care must be taken not to use any jelly (eg K.Y jelly) or disinfectant (eg.savlon) before doing pH test. Contact with cervical mucus must be avoided since it has a higher pH. The normal vaginal pH is 4.0. In BV, the pH is generally elevated to more than 4.5.

The vaginal pH test has the highest sensitivity (true negative) of the four characteristics used for identification of BV, but the lowest specificity (true positive); an elevated pH is also observed if the vaginal fluid is contaminated with menstrual blood, cervical mucus or semen, and in women with a *T. vaginalis* infection. In simple words it means that if pH test is negative the result can be taken as it is but if it is positive one has to rule out the other factors contaminating the sample such as menstrual blood, cervical mucus or semen or presence of *T. vaginalis* infection

Wet mount microscopy

Wet mount microscopy is the direct microscopic examination of vaginal discharge for the diagnosis of trichomoniasis, candidiasis and BV.

Box A1.1: Wet mount microscopy examination of vaginal discharge

Collect specimen	Take a specimen of discharge with a spatula from the side walls or deep in the vagina where discharge accumulates.
Prepare slide	Mix specimen with 1 or 2 drops of saline on a glass slide and cover with a cover slip.
What to look for	<ul style="list-style-type: none">Examine at 100X magnification and look for typical jerky movement of motile trichomonads (ovoid, globular, pear-shaped flagellated protozoan).
	<ul style="list-style-type: none">Examine at 400X magnification to look for yeast cells (round to ovoid cells with typical budding) and trichomonads.
	<ul style="list-style-type: none">To make identification of yeast cells easier in wet mount slides, mix the vaginal swab in another drop of saline and add a drop of 10% potassium hydroxide to dissolve other cells and note any fishy odour.
	<ul style="list-style-type: none">Presence of clue cells (squamous epithelial cells covered with many small coccobacillary organisms). Wet mount shows stippled granular cells without clearly defined edges because of the large numbers of adherent bacteria present and an apparent disintegration of the cells. The adhering bacteria are predominantly <i>G. vaginalis</i>, sometimes mixed with anaerobes).
Important	Look for evidence of other vaginal or cervical infections as multiple infections are common.



Fig A1b : Potassium hydroxide preparation of vaginal fluid showing budding yeast and mycelia

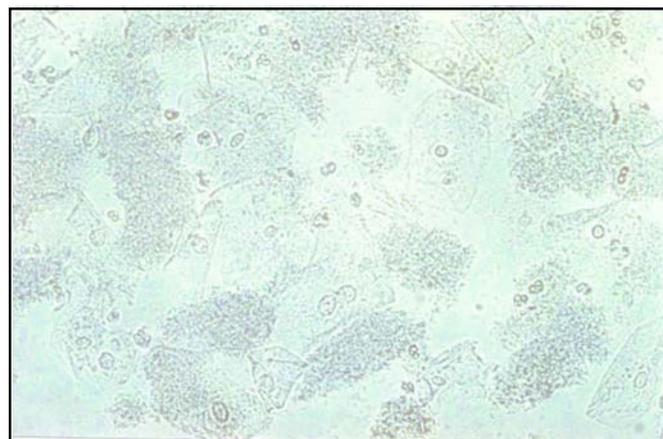


Fig A1c : "Clue cells" in vaginal wet mount (x 400)

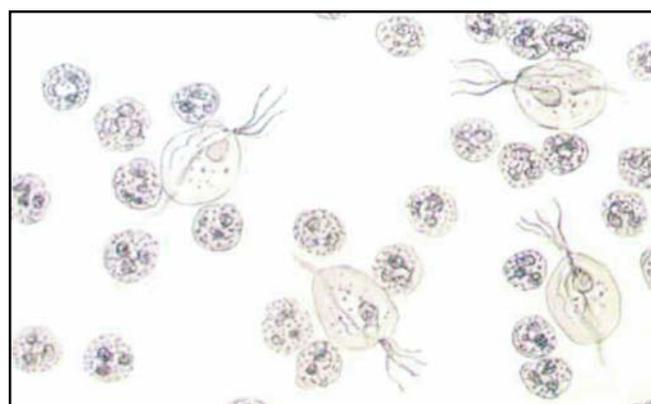


Fig A1d: *Trichomonas vaginalis* in a wet mount of vaginal discharge (x 400)

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Box A1.2: Clinical criteria for Bacterial vaginosis (BV): BV can be diagnosed using simple clinical criteria with or without the aid of a microscope.

Collect specimen	Note color and consistency of discharge. Take a specimen of discharge from the side walls or deep in the vagina where discharge pools (or use discharge remaining on speculum). Touch pH paper to discharge on swab or speculum and note pH.
Prepare slide	<ul style="list-style-type: none">• Place specimen on a glass slide. Add a drop of 10% potassium hydroxide (KOH) and note for any fishy smell.• Make a wet smear with 0.9% normal saline, cover with coverslip and see under microscope for clue cells.
What to look for	The diagnosis of BV is based on the presence of at least 3 of the 4 following characteristics <ul style="list-style-type: none">• Homogeneous white-grey discharge that sticks to the vaginal walls• Vaginal fluid pH >4.5• Release of fishy amine odour from the vaginal fluid when mixed with 10% potassium hydroxide (positive whiff test)• “Clue cells” visible on microscopy on wet preparation
Important	Look for evidence of other vaginal or cervical infections as multiple infections are common.

Whiff test

Women with BV often complain of a foul vaginal smell. This odour is due to the release of amines, produced by decarboxylation of the amino acids lysine and arginine by anaerobic bacteria. When potassium hydroxide is added to the vaginal fluid, these amines immediately become volatile, producing the typical fishy odour.

Place a drop of vaginal fluid on a glass slide and add a drop of 10% potassium hydroxide. Hold the slide close to nose to detect the amine odour. After a positive reaction, upon standing the specimen will quickly become odourless because the amines will be rapidly and completely volatilized.

Gram stain microscopy

A gram stain of a vaginal smear has a higher specificity for the detection of clue cells than a wet mount preparation. Moreover, a Gram stain allows good evaluation of the vaginal bacterial flora. Normal vaginal fluid contains predominantly Lactobacillus species and exceedingly low numbers of streptococci and coryneform bacteria. In BV, lactobacilli are replaced by a mixed flora of anaerobic

bacterial morphotypes and *G. vaginalis*. However, gram stain microscopy has a very low sensitivity for detecting gonorrhoea among women; culture remains the method of choice.

For men, gram stain microscopy of urethral discharge smear will show gram-negative intracellular diplococci in case of gonorrhoea. In case of non-gonococcal urethritis more than 5 neutrophils per oil immersion field (1000X) in the urethral smear or more than 10 neutrophils per high power field in the sediment of the first void urine are observed.

Box A1.3 Gram stain microscopy of vaginal smears

Collect specimen	A Gram stain slide can be prepared at the same time as the wet mount by rolling the spatula/swab on a separate slide.
Prepare slide	<ol style="list-style-type: none"> 1. Heat fix. 2. Stain with crystal violet (60 seconds) and rinse. 3. Stain with iodine (60 seconds) and rinse. 4. Decolorize with acetone-ethanol for few seconds (until the liquid runs clear). 5. Stain with safranin (60 seconds) and rinse. 6. Gently blot dry and examine under oil immersion (1000X) and count each type of organisms.
What to look for	<ol style="list-style-type: none"> 1. Lactobacilli only: Normal 2. Mixed flora, mainly lactobacilli with a few short rods (cocci): Considered normal 3. Presence of clue cells; mixed flora, mainly Gardnerella and anaerobic bacteria with a few lactobacilli diagnose as BV 4. Presence of clue cells, mixed flora of Gram-positive, Gram-negative and Gram-variable rods; no lactobacilli diagnose as BV 5. Count each type of organism and use the Nugent score to record the infection.
Important	Look for evidence of other vaginal or cervical infections as multiple infections are common.

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Box A1.4: *Nugent score

Scoring system (0 to 10) from Gram-stained vaginal smears^a

Total Score	Lactobacillus morphotypes	Gardnerella and Bacteriodes spp. morphotypes	Curved gram-variable rods
0	4 +(>30/oif)	0(0/oif)	0
1	3 +(5-30/oif)	1 +(<1/oif)	1+ or 2+
2	2 +(1-4/oif)	2 +(1-4/oif)	3+ or 4+
3	1 + (<1/oif)	3 +(5-30/oif)	
4	0 (0/oif)	4 +(>30/oif)	

^aMorphotypes are scored as the average number seen per oil immersion field(oif). Note that less weight is given to curved Gram - variable rods. Total score = lactobacilli + *G. vaginalis* and *Bacteriodes* spp. + curved rods.

0 = no morphotypes present

1 = <1morphotypes present

2 = 1 to 4 morphotypes present

3 = 5 to 30 morphotypes present

4 = 30 or more morphotypes present.

Interpretation of Nugent score

0-3 = normal, never treat

4-6 = intermediate, decide on symptoms for treatment

7-10 = Treat

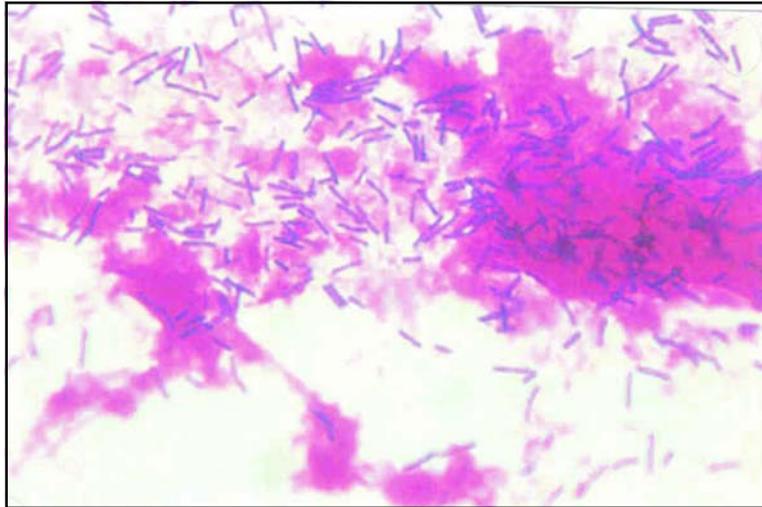


Fig A1e: Gram stained vaginal smear showing a normal flora of lactobacilli (x 1000)

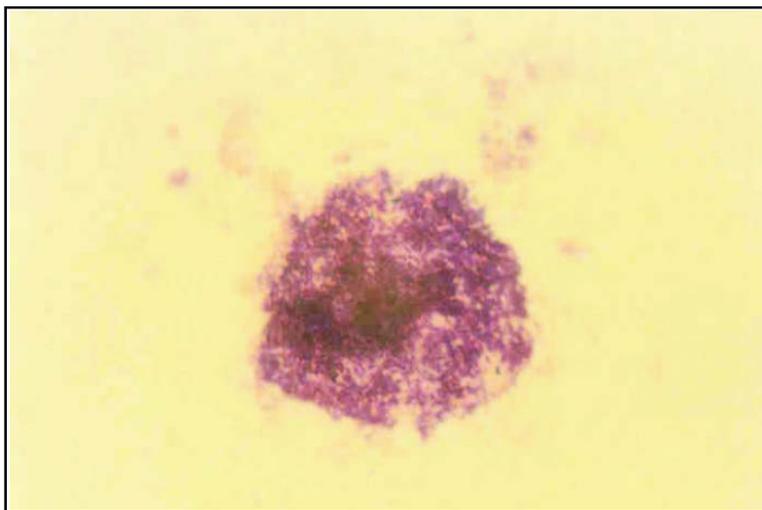


Fig A1f: Gram stained vaginal smear with typical "clue cell" (x 1000)

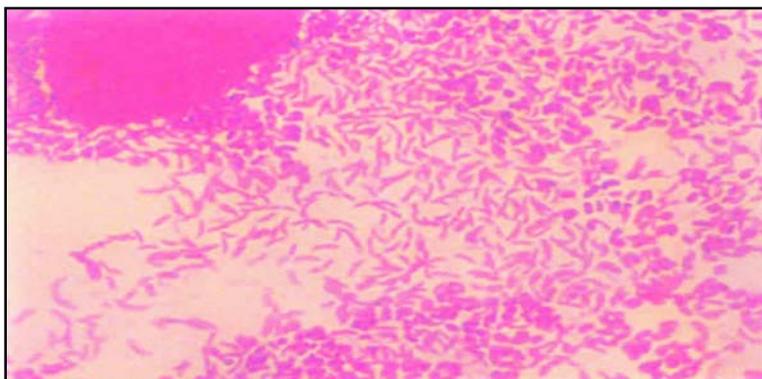


Fig A1g: Gram stained vaginal smear showing large Gram- negative rods (*Mobilincus mulieris*) (x 1000)

Annexure-1

Use of gram stain for diagnosis of cervical infection

1. The Gram stain method in female does not provide conclusive evidence of the presence of Gonococcal infection. Presence of gram negative diplococci indicates infection but their absence does not rule out infection.
2. The costs associated with the method, including the cost of maintaining microscopes, outweigh the benefits in terms of improved quality of care.

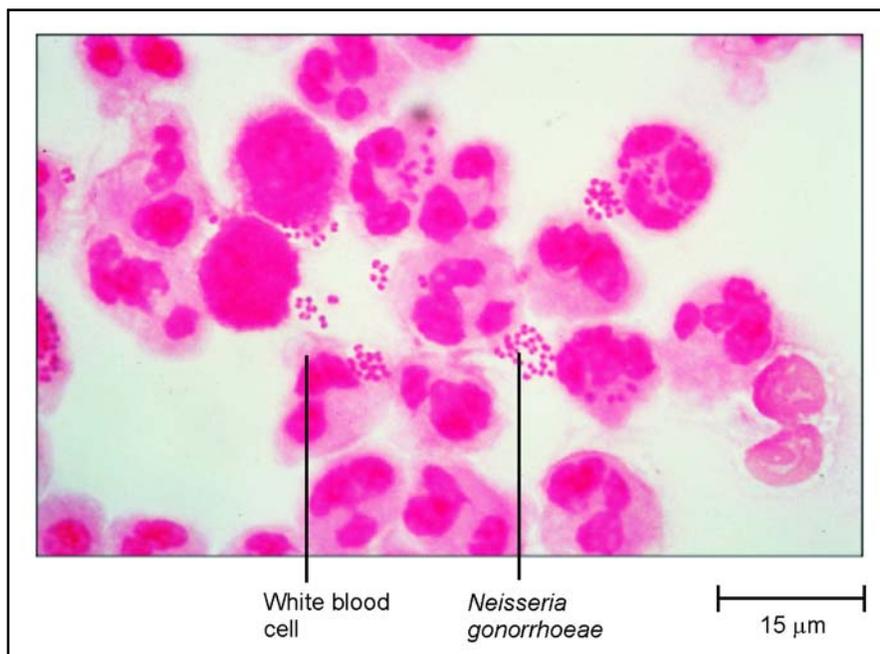


Fig A1h: Gram stain smear - Gram-negative diplococci of *Neisseria gonorrhoeae*

Rapid Plasma Reagin (RPR) test for Syphilis

The current nontreponemal tests for syphilis are Venereal Disease Research Laboratory (VDRL) and rapid plasma reagin (RPR) test. RPR test is most suitable for the primary health care set-up.

Box A1.5 : Procedure of RPR test

Procedure of RPR test

- Inform about the infection and the procedure for diagnosis
- Seek consent
- Use a sterile needle and syringe. Draw 5 ml of blood from a vein. Put in a plain test tube
- Let the test tube stand for 20 minutes to allow serum to separate (Or centrifuge 3–5 minutes at 2000–3000 rpm). In the separated sample, serum will be on top.
- Use sampling pipette to transfer the serum. Take care not to include any red blood cells from the lower part of the separated sample.
- Hold the pipette vertically over a test card circle. Squeeze test to allow one drop (50 μ l) of serum to fall onto a circle. Spread the drop to fill the circle using a toothpick or other clean spreader.

Important: Several samples may be done on one test card. Be careful not to contaminate the remaining test circles. Use new tip and spreader for each sample. Carefully label each sample with a Client name or number

- Attach dispensing needle to a syringe. Shake antigen.* Draw up enough antigen for the number of tests done (one drop per test).
- Holding the syringe vertically, allow exactly one drop of antigen to fall onto each test sample. Do not stir.
- Rotate the test card smoothly on the palm of the hand for 8 minutes (or rotate on a mechanical rotator.)

Interpreting results

After 8 minutes rotation, inspect the card in good light. Turn or tilt the card to see whether there is clumping (reactive result). Test cards include negative and positive control circles for comparison.

Example test card	<ol style="list-style-type: none"> 1. Non-reactive (no clumping or only slight roughness): Negative for syphilis 2. Reactive (highly visible clumping): Positive for syphilis 3. Weakly reactive (minimal clumping): Positive for syphilis <p>Note: Weakly reactive can also be more finely granulated and difficult to see than this illustration</p>
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* Make sure antigen was refrigerated (not frozen) and has not expired.

If RPR positive:

- Enquire if the woman and her partner have received proper treatment.
- If not, treat woman and partner for syphilis with benzathine penicillin.
- Treat newborn with benzathine penicillin.
- Follow-up newborn in 2 weeks.
- Counsel on safer sex.

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Correlation and confirmation of test results

- Syphilis tests detect antibodies, which are evidence of current or past infection. Syphilis tests are not needed to diagnose Clients with genital ulcers (who should be managed using Flowchart).
- Non-treponemal tests (such as RPR and VDRL) are the preferred tests for screening. These tests detect almost all cases of early syphilis, but false positives are possible. RPR can be performed without a microscope.

Treponemal tests, such as *Treponema pallidum* haemagglutination test (TPHA), fluorescent *Treponema* antibody absorption test (FTA-Abs), microhaemagglutination assay for antibodies to *Treponema pallidum* (MHA-TP), if available, can be used to confirm non-treponemal test results.

Quantitative RPR titres can help evaluate the response to treatment.

The following table can be used to interpret syphilis test results.

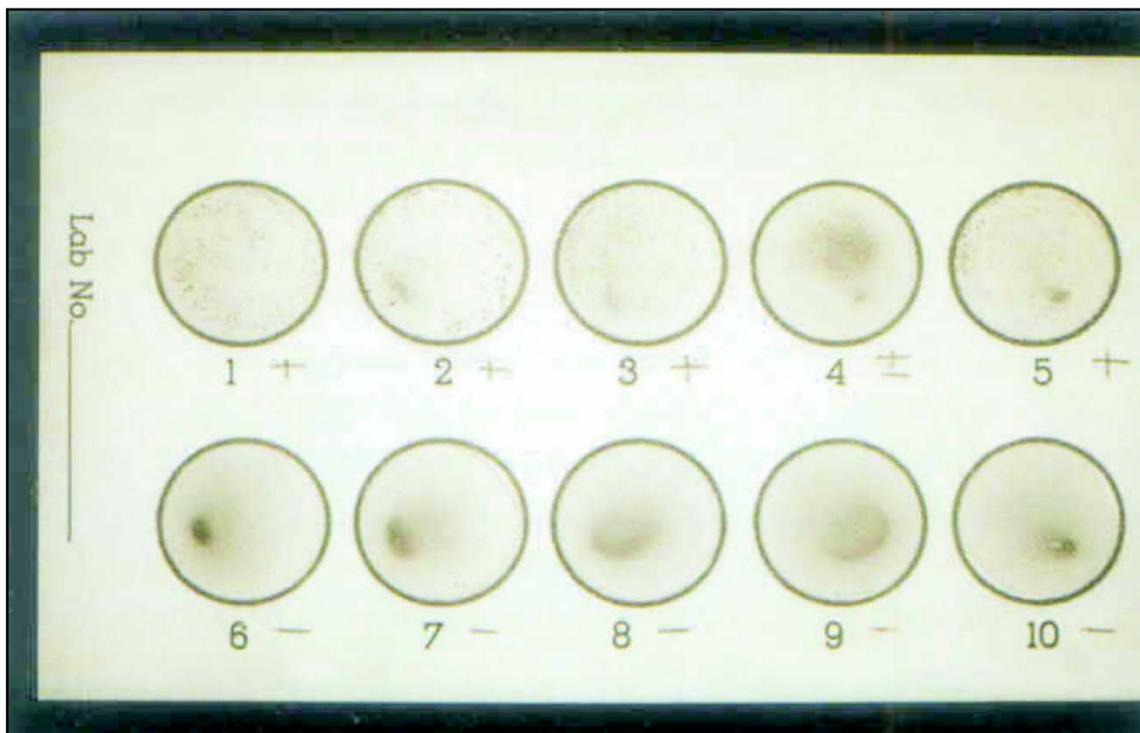
Note: where additional tests are not available, all Clients with reactive RPR or VDRL should be treated.

Box A1.6: Interpreting serological test results

	RPR	RPR titre	TPHA
Active infection	+	>1:8	+
Latent syphilis	+	Often <1:4	+
False positive	+	Usually <1:4	-
Successful treatment	+ or -	2 titres decrease (e.g. from 1:16 to 1:4)	+



FigA1i : Test serum is mixed with antigen and the card is placed on appropriate rotator



FigA1j: Reading RPR results for 10 undiluted sera showing reactive and non reactive samples. The presence of small to large flocculated clumps indicates reactivity, whereas no clumping or a very slight roughness indicates non-reactivity

