Examination of Sputum

Sputum examination refers to the examination of the material coughed out from the lungs, bronchi, trachea, and larynx. Normally, sputum is composed predominantly of mucus and also certain cellular and non-cellular components of host origin. During expectoration, sputum gets contaminated with cells and normal bacterial flora from the mouth and pharynx.

Examination of sputum is mainly carried out for:

- **Identification of the causative organism** in a suspected infection of the lower respiratory tract, e.g.
  - Pneumonia especially if severe or in an immunocompromised host.
  - Suspected tuberculosis
  - Suspected fungal infection
  - *Pneumocystic carinii* pneumonia in HIV-positive patients
  - Infective exacerbation of a chronic disease like bronchiectasis
- **Cytologic examination** for malignant cells, investigation of asbestosis, investigation of viral infections (viral inclusions in cytomegalovirus and herpes simplex infections) and fungal infections.

COLLECTION OF SPUTUM

1. Sputum sample is preferably collected in the morning (since secretions accumulate overnight), soon after awakening and before taking any mouthwash or food.
2. Sample is collected in a clean, dry, wide-mouthed container with a securely fitting screw cap. The container should be leak proof and break-resistant to prevent aerosol formation and desiccation, and should have the capacity of about 25 ml.
3. Patient takes a deep breath 2-3 times filling his/her lungs, coughs deeply, and spits into the container. About 2-5 ml of sputum is collected. Sample consisting only of saliva (foamy, clear, and watery appearance) is not acceptable; in such a case, another sample should be obtained. The container is capped securely and labeled.

Induction of Sputum

If the patient is unable to bring out the sputum spontaneously, inhaling aerosol of 15% sodium chloride and 20% propylene glycol for 20 minutes can induce expectoration. Sputum can also be induced by inhaling nebulized hypertonic saline or distilled water in association with chest physiotherapy.

For microbiologic studies, sample should be sent to the laboratory immediately. If sputum is allowed to stand, rapid proliferation of contaminating bacterial flora from oral cavity and throat will occur leading to incorrect results. In addition, pathogenic organisms, **especially *Haemophilus influenzae*, do not survive in collected samples for long.** Sample for bacterial culture should not be refrigerated.

If sample is to be transported to a distant laboratory for mycobacterial culture, sputum should be collected in 25 ml of following solution:

- N-acetylpyridinium chloride 5 gm
- Sodium chloride 10 gm
- Distilled water upto 1000 ml

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APPEARANCE OF SPUTUM

Appearance of sputum is often suggestive of the underlying pathologic process as follows:

- Bloody: Hemoptysis (pulmonary tuberculosis, lung abscess, bronchiectasis, bronchogenic carcinoma, mitral stenosis, pulmonary infarction)
- Rusty: *Pneumococcal lobar pneumonia*
- Bloody and gelatinous (red current jelly): *Klebsiella* pneumonia
- Green: *Pseudomonas* infection
- Purulent and separating into 3 layers on standing: Bronchiectasis, lung abscess
- Pink, frothy (air bubbles): Pulmonary edema
- Copious amounts of purulent sputum: Lung abscess, bronchiectasis, bronchopleural fistula

MICROBIOLOGICAL EXAMINATION

Sputum sample is often contaminated with normal flora of the oral cavity and pharynx (Box 8.1).

Box 8.1: Normal flora of the oral cavity and pharynx

Gram-positive: Staphylococci (*S. aureus*, *S. epidermidis*), streptococci (*S. viridans*, *S. pneumoniae*), *Diptheroids*, enterococci, micrococci, lactobacilli, Yeasts (*Candida* spp.).

Gram-negative: *Neisseria* spp; *Haemophilus* spp; fuso- bacteria, coliforms, *Moraxella catarrhalis*.

Gram Staining

Pathogenic organisms found in sputum include:

1. **Gram-positive**: *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*.
2. **Gram-negative**: *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Yersinia pestis*, *Moraxella catarrhalis*.

For bacteriologic examination, sputum sample should be processed in the laboratory within 1 hour of collection. The sample is transferred to a sterile Petri dish and its appearance is noted. A thin smear is made on a glass slide from the purulent portion of the sputum with a clean stick, air-dried, fixed, and stained with Gram's stain. Purely mucoid, watery, frothy, or white samples usually show squamous epithelial cells covered with masses of bacteria; this indicates that the sample consists mainly of secretions from the mouth and the throat. Such samples are not suitable for bacteriological examination (Fig. 8.1). If polymorphonuclear neutrophils are less than 10 per epithelial cell, culture is not carried out.

Gram stained smear of sputum should be interpreted carefully because of the presence of various contaminating gram-positive and gram-negative organisms originating from mouth and throat (normal bacterial flora).

Morphological appearance on Gram stained smear is suggestive of a particular organism as follows:

- Gram-positive diplococci with surrounding clear space (capsule): *S. pneumoniae* (Fig. 8.2).
- Gram-positive cocci in grape-like clusters: *S. aureus*.

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- Gram-positive yeast cells with budding and pseudohyphae: *Candida*.
- Gram-negative diplococci, both intra- and extra- cellular: *Moraxella catarrhalis*.
- Gram-negative coccobacilli: *H. influenzae*.
- Large granules with center gram-negative and periphery gram-positive: *Actinomyces*.

Bacteriological Culture

A floccule of purulent portion of sputum is inoculated on culture media for definitive identification of organisms. Sputum sample is considered as unsatisfactory for culture if it contains >25 squamous cells/low power field. Ideal sputum sample for culture contains alveolar macrophages, numerous neutrophils (>5/high power field), bronchial epithelial cells, and few squamous cells (<10/high power field). To reduce the amount of contaminating normal bacterial flora in the inoculum, saliva is washed away from sputum with sterile normal saline. **The washed sputum is inoculated on (i) blood agar plate and (ii) chocolate agar (heated blood agar). The blood agar plate is incubated aerobically and chocolate agar plate is incubated in an atmosphere of extra carbon dioxide.** Inoculated plates are inspected for growth after incubation for 18 hours; if growth is not satisfactory, incubation for further 24 hours is indicated. Antibiotic sensitivity test is carried out only if amount of growth is significant.

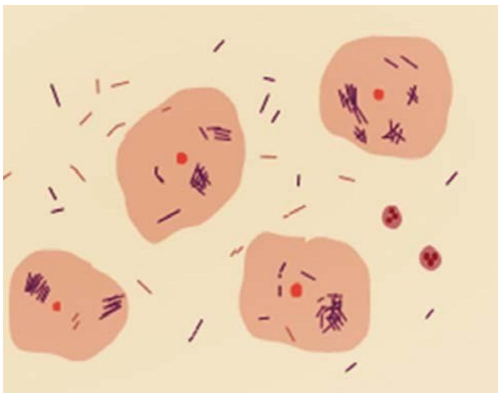

Fig. 8.1: Unacceptable sputum sample: Sputum sample shows many squamous cells covered with masses of bacteria

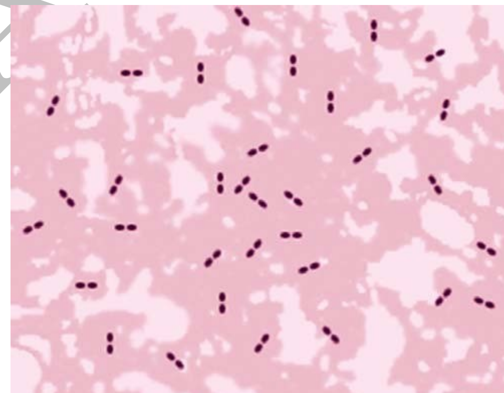

Fig. 8.2: Gram stained smear of sputum showing gram-positive diplococci (*Streptococcus pneumoniae*)

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<u>EXAMINATION OF SPUTUM FOR MYCOBACTERIUM TUBERCULOSIS</u>

Tuberculosis is one of the major public health problems in many different countries. Early diagnosis of pulmonary tuberculosis will lead to early institution of therapy enabling cure, and also prevention of spread of disease to others.

Mycobacterium tuberculosis complex comprises of *M. tuberculosis*, *M. bovis*, and *M. africanum*. These tubercle bacilli are the aetiologic agents of human tuberculosis. Other mycobacteria are called as non-tuberculous.

There are two main approaches for diagnosis of tuberculosis:

- **Direct tests:** This consists of detection of *M. tuberculosis* or its components
- **Indirect tests:** This involves detection of humoral or cellular immune response to tuberculous infection.
- **Direct tests** for diagnosis of tuberculosis on sputum sample are as follows:
- Examination of sputum smear
  1. Ziehl-Neelsen technique
  2. Fluorescence microscopy
- Culture on conventional media
- Commercial automated culture systems
- Molecular methods.

Examination of Sputum Smear

For detection of *M. tuberculosis*, at least **three sputum samples** collected on three separate occasions (including at least one early morning specimen) need to be examined. A thin smear is prepared on a clean glass slide from blood-tinged, opaque, grayish, or yellowish portion. Children often swallow sputum and may not be able to cough it up; in them a sample of fasting gastric juice can be aspirated and examined like sputum.

The smear is stained with Ziehl-Neelsen stain and examined under the ordinary light microscope. If fluorescent microscope is available, smear can be examined after staining it with a fluorochrome **(auramine-rhodamine or auramine O).**

Ziehl-Neelsen stain of sputum smear: This simple, inexpensive, and rapid technique is mainly useful for:

- Diagnosis of infectious cases of pulmonary tuberculosis. (Sputum smear-positive cases are a major source of spread of infection).
- Assessment of response to anti-tuberculous treatment.
- Determining cure or treatment failure.

Ziehl-Neelsen-stained sputum smear is positive if at least 5000-10000 tubercle bacilli/ml are present in the sputum. Sensitivity of the technique is reported to be 60- 80%. Chances of detection of tubercle bacilli are increased if multiple sputum samples are examined or if bleach concentration technique is used. In bleach concentration technique, a **solution of bleach** (concentrated sodium hypochlorite) is added to the sputum sample, which leads to **the liquefaction of mucus and killing of mycobacteria**. After centrifugation (or overnight sedimentation), smears are prepared from the sediment, stained, and examined.

With Ziehl-Neelsen staining, **mycobacteria appear as bright red straight** or slightly curved beaded **rods (2-4 μ in length and 0.2-0.5 μ in width) against a blue or green background (Fig. 8.3). Mycobacteria are both acid- and alcohol-fast and are termed acid-fast bacilli (AFB).** If acid- fast bacilli are seen, their number should be reported. At least 100 fields are examined before reporting the smear as negative.

A negative smear does not rule out the diagnosis of tuberculosis since organisms may be few in number, sputum sample may not have been collected satisfac- torily, or smear may be of poor quality.

Fluorescence microscopy: *M. tuberculosis* can also be stained with a fluorochrome (auramine-rhodamine or auramine O) and the preparation is examined under fluorescence microscope. Mycobacteria **fluoresce bright yellow** against a dark

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background (Fig. 8.4). This technique is rapid (since the smear is examined under low power) and is especially helpful if organisms are few in number. It is necessary to confirm a positive smear by Ziehl-Neelsen stain since false-positive rate is high.

Culture (Conventional)

The definitive diagnosis of tuberculosis is based on isolation of *M. tuberculosis* from culture of sputum sample. Culture is usually carried out for:

- Drug susceptibility testing
- Precise species identification, if organism other than

M. tuberculosis is suspected (for epidemiologic purpose).

- Diagnosis in patients who have typical clinical and radiological features of tuberculosis but are sputum smear-negative.

Culture is more sensitive than sputum smear examination as it can detect 10-100 microorganisms per ml of sputum sample. Its sensitivity for diagnosis of tuberculosis is 80-85% and specificity is 98%. However, culture is expensive, around 6 weeks are required for result and even longer for drug susceptibility testing, and prior decontamination of sputum is required to kill normal bacterial flora.

Sputum samples are contaminated to a varying degree with normal bacterial flora. These bacteria are rapidly growing and digest the culture medium before tubercle bacilli begin to grow. Therefore, before inoculation, digestion and decontamination of sputum sample is carried out to liquefy the organic debris so that the decontaminating agent can reach the bacteria, and kill the contaminating organisms.

Traditional culture media for isolation of *M. tuberculosis* are:

- *Solid media*: Egg-based (Lowenstein-Jensen medium) or agar-based (Middlebrook 7H10 or 7H11).
- *Liquid media*: Middlebrook 7H9, Middlebrook 7H12.

The most commonly used solid medium for culture is Lowenstein-Jensen medium. Visible mycobacterial growth may require up to 6 weeks. Further biochemical tests are required for determination of species.

Molecular Methods

There are two approaches for molecular diagnosis of tuberculosis in sputum samples:

- Direct detection of *Mycobacterium tuberculosis* in sputum sample
- Detection of *Mycobacterium tuberculosis* in isolates from culture by nucleic acid probes.

M. tuberculosis can be rapidly identified directly in sputum samples by detecting DNA sequences specific to it. A commonly used DNA target for this purpose is **IS 6110** that is observed only in *M. tuberculosis* complex. Multiple copies of this sequence are present in the genome of *M. tuberculosis*. **This method can detect 10-1000 organisms per ml of sputum**. Other DNA and RNA sequences specific for *M. tuberculosis* complex can also be targeted.

Laboratory cross-contamination (due to aerosolized PCR product) is responsible for significant number of false-positive results. This test is also expensive. PCR amplifies DNA sequences of both live and dead bacilli. Therefore, the test cannot be used to assess response to therapy.

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PCR-based assays should be interpreted in the light of clinical features, prevalence of tuberculosis in the population, and findings on Ziehl-Neelsen smear.

EXAMINATION FOR OTHER ORGANISMS

Additional investigations given below are indicated when infection by following organisms is suspected:

- *Pneumocystis carinii*: Bronchoalveolar lavage fluid stained with silver stain (Fig. 8.5) and Giemsa stain
- *Yersinia pestis* (pneumonic plague): Giemsa smear
- Yeast-like organisms on Gram's smear: Sabouraud dextrose agar
- Histoplasmosis: Giemsa smear
- *Aspergillus*: Potassium hydroxide wet mount of sputum
- *Paragonimus*: Saline wet mount of sputum for eggs.

CYTOLOGICAL EXAMINATION OF SPUTUM

Cytological examination of sputum is usually carried out for investigation of bronchogenic carcinoma. Sometimes, it may also be helpful in the identification of viral inclusions (like those of *cytomegalovirus* and *Herpes simplex* virus), fungi, protozoa, and asbestos bodies.

For cytological examination, fresh early morning sputum sample is preferred. **For detection of lung cancer**, it is recommended to collect sputum sample daily for five consecutive days. This is because multiple sputum samples increase the chances of detection of malignant cells (Fig. 8.6).

Sputum sample may be either spontaneously produced or artificially induced. If patient is unable to produce sputum spontaneously by deep coughing, he is made to inhale a heated, aerosolized solution of 15% sodium chloride and 20% propylene glycol for 20 minutes. This usually results in induction of a satisfactory sputum sample.

Ideally, freshly collected sputum should be sent immediately to the laboratory without addition of any fixative. If delay is anticipated, prefixation of sputum with **Saccomano's fixative** is recommended. This consists of collection of sputum in a mixture of 50% ethyl alcohol and 2% carbowax.

In the laboratory, smears are made from blood-tinged portion or from tissue fragments in sputum and stained with Papanicolaou technique. Sputum sample is considered as adequate for cytological evaluation if alveolar macrophages or bronchial epithelial cells are seen in the smear.

The average sensitivity of sputum examination for detection of malignant cells is about 65%. Sensitivity increases if:

- Increased numbers of sputum samples are examined
- Lesion is centrally located rather than at the periphery of the lung
- Size of tumor is large
- Histologic type of carcinoma is of squamous nature rather than adenocarcinoma or small cell carcinoma.

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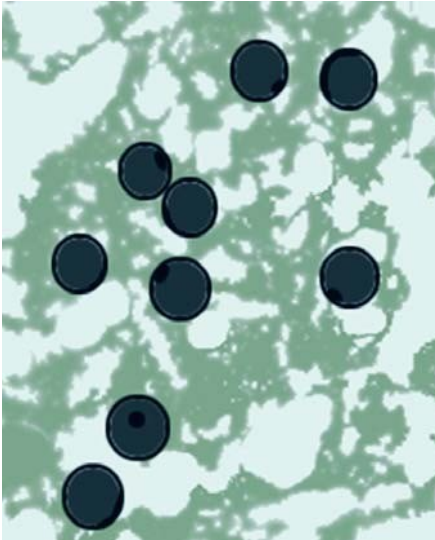

Fig. 8.5: *Pneumocystis carinii* cysts in bronchoalveolar lavage fluid (silver methenamine stain)

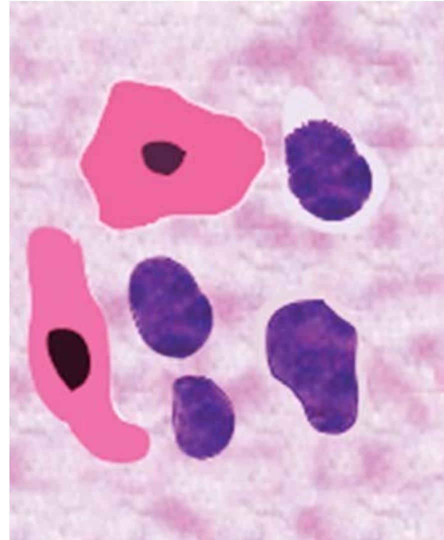

Fig. 8.6: Sputum examination showing malignant cells of squamous type

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