Examination of Sputum

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

Examination of sputum is mainly carried out for:

- **Identification of causative agent or organism** associated with a particular suspected infection of the lower respiratory tract, e.g.
  - Suspected tuberculosis
  - Pneumonia especially if severe or in an immunocompromised host
  - Pneumocystic carinii pneumonia in HIV-positive patients
  - Suspected fungal infection
  - Infective exacerbation of a chronic disease like bronchiectasis

- **Cytological examination** for the investigation of viral infections (viral inclusions in *cytomegalovirus* and *herpes simplex* infections), fungal infection, asbestosis and malignant cells.

1. Sputum sample is ideally collected in the morning (since secretions accumulate overnight), soon after awakening and before taking any mouthwash or food.
2. Sputum sample is collected in a sterile, clean, dry and wide-mouthed plastic container with a securely fitting screw cap. The container should be of unbreakable or break-resistant plastic and leak-proof to prevent desiccation and aerosol formation, and should have the capacity of about 30 ml.
3. The patient is advised to take a deep breath 2-3 times filling his/her lungs, coughs deeply, and spit into the plastic container. About 2-5 ml of sputum is collected. Sample consisting the only of saliva (watery appearance, clear, and foamy) is not acceptable for laboratory investigations; in such case, another sample should be collected. The container, containing sputum sample, is capped securely and labeled properly.

**Induction of Sputum**

If the patient is not able to expectorate the sputum spontaneously, inhaling aerosol of 15% sodium chloride (NaCl) and 20% propylene glycol (C₃H₈O₂) for 20 minutes can induce expectoration. Sputum can also be induced by inhaling distilled water in...
association with chest physiotherapy or by inhaling nebulized hypertonic saline.

For microbiological examination of sputum, sample should be sent to the laboratory immediately. If sputum is allowed to stand, rapid reproduction of contaminating bacterial flora from the throat and oral cavity will occur leading to incorrect results. Inclusion, pathogenic organism, especially *Haemophilus influenzae*, do not survive for a long time in the collected sample. Sputum sample for bacterial culture should not be refrigerated.

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

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

**APPEARANCE OF SPUTUM**

Physical appearance of sputum is often indicative and symptomatic of the underlying pathologic process as follows:

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

**MICROBIOLOGICAL EXAMINATION OF SPUTUM**

Sputum sample is usually adulterated and contaminated with normal flora of the pharynx and oral cavity. Normal flora found in the pharynx and oral cavity are listed below.

- **Gram-positive microorganisms**: *Diptheroids*, streptococci (*S. pneumoniae*, *S. viridans*), staphylococci (*S. epidermidis*, *S. aureus*), lactobacilli, enterococci, Yeasts (*Candida* spp.), micrococci.
- **Gram-negative microorganisms**: Coliforms, *Haemophilus* spp; *Neisseria* spp; *Moraxella* catarrhalis, fusobacteria.

**Gram staining**

Pathogenic organisms found in sputum include—

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

For bacteriological examination of sputum, sample should be processed in the laboratory within an hour our collection. A small
amount of sputum is transferred to a sterile Petri dish and its physical appearance is noted. From the purulent portion of the sputum, a thin smear is made on the grease-free sterile glass slide with a clean stick. The slide is air-dried, fixed and stained with Gram's stain. Entirely watery, mucoid, white, or frothy samples often show squamous epithelial cells covered with the bunches of bacteria; this phenomenon indicates that the sample consists mainly of secretions from the mouth and the throat. Such samples are not acceptable for bacteriological examination (see Figure 989.1). Culture is not carried out if polymorphonuclear neutrophils are less than 10 per epithelial cell.

Because of the presence of various contaminating Gram-positive and Gram-negative microorganism deriving from throat and mouth (normal bacterial flora), Gram-stained smear of sputum should be elucidated carefully.

Morphological appearance of bacterial cells on Gram stained smear is redolent of a particular microorganism as follows:

- **Gram-negative diplococci, both intra- and extracellular**: Moraxella catarrhalis.
- **Gram-positive yeast cells with budding and pseudohyphae**: Candida.
- **Gram-positive diplococci with surrounding clear space (capsule)**: S. pneumoniae (see Figure 989.2).
- **Gram-negative coccobacilli**: H. influenzae.
- **Gram-positive cocci in grape-like clusters**: S. aureus.
- **Large granules with center gram-negative and periphery gram-positive**: Actinomyces.

Bacteriological Culture

Culture media is inoculated with a floccule of the purulent portion of sputum for absolute identification of microorganism. Sputum sample is considered as unsuitable for the bacterial culture if it contains >25 squamous epithelial cells/low power field. An ideal sputum sample for bacterial culture contains bronchial epithelial cells, numerous neutrophils (>5/high power field), alveolar macrophages, and few squamous epithelial cells (<10/high power field). Saliva is washed away from sputum with sterile
normal saline in order to reduce the amount of contaminating normal bacterial flora in the inoculum. Blood agar plate and chocolate agar (heated blood agar) are inoculated with the washed sputum. The chocolate agar plate is incubated in an atmosphere of extra carbon dioxide (CO₂) and blood agar plate is incubated aerobically. After the incubation for 18 hours, inoculated agar plates are examined for growth; if growth is not sufficient, incubation for further 24 hours is indicated. Antibiotics sensitivity test is carried out only if the amount of bacterial growth is significant.

1. **Direct test:** This involves detection of *M. tuberculosis* or its components
2. **Indirect test:** This consists of detection of cellular or humoral immune response to tuberculosis infection.

**Direct tests for the detection of tuberculosis on sputum sample are as follows:**

1. Examination of sputum smear
   - Ziehl-Neelsen technique
   - Fluorescence microscopy
2. Molecular Method
3. Culture on standard media
4. Commercial automated culture system

**Examination of Sputum Smear**

For detection of *M. tuberculosis*, minimum three sputum samples collected on three different occasions (including at least one early morning sputum sample) need to be examined. A thin sputum smear is prepared on clean, sterile, grease-free glass slide from a yellowish, grayish, opaque, or blood-tinged portion of sputum. Children often ingest sputum and may be unable to cough it up; in such condition sample of fasting gastric juice can be aspirated and examined like sputum.

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

**Ziehl-Neelsen stain of sputum smear:** This technique is very simple, rapid and inexpensive. This technique is mainly used for:
Examination of Sputum

- Diagnosis of pulmonary tuberculosis. (A positive sputum smear cases are the major source of spread of infection).
- Determining cure or treatment failure.

Ziehl-Neelsen-stained sputum smear is considered as positive if 5000-10000 tubercle bacilli/ml are present in the sputum. Sensitivity of the technique is reported to be 60-80%. Possibilities 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 concentrated sodium hypochlorite (NaOCl) is added to the sputum sample, which causes the liquefaction of mucus and killing of mycobacteria. The sample is kept for overnight sedimentation (or centrifugation), from the sediment of sputum a thin smear is prepared, stained and examined.

With Ziehl-Neelsen staining, mycobacteria appear as bright red straight or slightly curved rods (0.2-0.5 μ in width and 2-4 μ in length) against a green or blue background (see Figure 989.3). Mycobacteria, both acid- and alcohol-fast are termed as acid-fast bacilli (AFB). Minimum 100 fields are examined before reporting the smear as negative. If acid-fast bacilli are seen, their number should be reported.

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

Fluorescence microscopy: A thin sputum smear is prepared and stained with a fluorochrome (auramine O or auramine-rhodamine). The smear is examined under fluorescence microscope. Mycobacteria appear as bright yellow against a dark background (see Figure 989.4). This technique is very simple and rapid (since the sputum smear is examined under low power lens) and this technique is very useful if the organisms are few in numbers. It is very necessary to confirm a positive sputum smear with Ziehl-Neelsen stain since there is a high rate of false-positive result.
**Molecular Method**

There are two different methods for the molecular diagnosis of tuberculosis in sputum samples:

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

*M. tuberculosis* can be rapidly detected directly in sputum samples by identifying DNA sequences specific to it. In *M. tuberculosis* complex, **IS 6110** is the targeted DNA because it is only observed in *M. tuberculosis* complex. In the genome of *M. tuberculosis*, multiple copies of this sequence are present. By this method, 10-1000 organisms per ml of sputum can be detected. Other DNA and RNA sequences precise for *M. tuberculosis* complex can also be targeted.

Laboratory cross-contamination (due to aerosolized PCR products) is also responsible for unreliable and false-positive results. PCR amplifies DNA sequences of both dead and live bacilli, for that reason the test cannot be used to evaluate response to therapy. This test is also expensive. PCR-based assays should be elucidated in the light of clinical features, findings on Ziehl-Neelsen sputum smear and presence of tuberculosis in other family members.

**Sputum Culture (Conventional)**

For the definitive diagnosis of tuberculosis, pure culture technique is used. *M. tuberculosis* is isolated from the culture of sputum sample. Sputum culture is usually carried out for:

- Identification of a particular species, if organism other than *M. tuberculosis* is suspected (for the purpose of incidence, distribution, and control of diseases).
- Drug susceptibility testing.
- Diagnosis in patients who have distinctive radiological and clinical features of tuberculosis but are sputum smear-negative.

Sputum culture is more sensitive as compared to sputum smear examination. It can detect 10 to 100 microorganism in per ml of sputum sample. Its sensitivity for the identification of tuberculosis is 80-85% and specificity is 98%. However, this procedure is very expensive but reliable, around 6 weeks are needed for the result and even longer for the drug susceptibility testing, and earlier decontamination of sputum is required to kill normal bacterial flora.

Contaminating bacteria grows rapidly and digest the culture medium prior tubercle bacilli begin to grow. Therefore, it is necessary to decontaminate the sputum sample by adding 4% sodium hydroxide (used as decontaminating agent).

Standard culture media for the isolation of *M. tuberculosis* are:

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

The most common solid medium used for the culture is Lowenstein-Jensen medium. Up to 6 weeks are required for the visible mycobacterial growth. For the identification of species, further biochemical tests are performed.
Commercial Automated Culture Systems

Nowadays, rapid automated culture systems are available commercially which can give results within two weeks (instead of six weeks with standard media). However, this procedure is expensive. Examples of such systems are BACTEC™ 460TB system (see Figure 989.5) and BACTEC™ 9050 automatic blood culture analyzer (Becton-Dickinson Diagnostic Instruments Systems, Maryland, USA). These instruments are very sensitive and can detect \textit{M. tuberculosis} in clinical samples. In this method, broth is used in which radiolabelled \( ^{14}\text{C}-\text{palmitate} \) has been integrated. Mycobacteria metabolize \( ^{14}\text{C}-\text{palmitate} \) to radiolabelled \( ^{14}\text{CO}_{2} \), which is further detected by the instrument.

- \textit{Paragonimus}: Saline wet mount of sputum for eggs.
- \textit{Histoplasmosis}: Giemsa smear.
- \textit{Pneumocystis carinii}: Bronchoalveolar lavage fluid stained with Giemsa stain and silver stain (see Figure 989.6).
- \textit{Yersinia pestis} (pneumonic plague): Giemsa smear.
- \textit{Aspergillus}: Potassium hydroxide wet mount of sputum.
- Yeast-like organisms on Gram’s smear: Sabouraud dextrose agar.

![BACTEC™ 460TB system](image)

Figure 989.5 BACTEC™ 460TB system

**CYTOLOGICAL EXAMINATION OF SPUTUM**

Cytological examination of sputum is normally carried out for the diagnosis of bronchogenic carcinoma. Occasionally, it may also be useful in the identification of fungi, protozoa, asbestos bodies and viral inclusions (like those of \textit{cytomegalovirus} and \textit{Herpes simplex} virus).

For cytological examination, early morning sputum sample is preferred. For the diagnosis of lung cancer, it is suggested to collect sputum sample daily for first five
successive days. This method will increase the chances of detection of malignant cells (see Figure 989.7).

![Figure 989.7 Sputum examination showing malignant cells of squamous type](image)

Sputum sample may be either spontaneously produced or artificially induced. If the patient is not able to expectorate the sputum spontaneously, inhaling aerosol of 15% sodium chloride (NaCl) and 20% propylene glycol (C₃H₈O₂) for 20 minutes can induce expectoration. This normally results in the induction of sufficient sputum sample.

The sputum sample should be sent to the laboratory just after the collection without the addition of any fixative. If the sample is to be transported to a remote laboratory, prefixation of sputum with Saccomano’s fixative is recommended. This involves collection of sputum in a mixture of 2% carbowax and 50% ethyl alcohol.

In the laboratory, a thin sputum smear is prepared on clean, sterile, grease-free glass slide from a yellowish, grayish, opaque, or blood-tinged portion, or from tissue fragments in sputum and stained with Papanicolaou technique. The sputum sample is considered as adequate for cytological examination bronchial epithelial cells or alveolar macrophages are seen in the smear.

The average sensitivity is about 65% in sputum examination for detection of malignant cells. Sensitivity increases as per following conditions:

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

REFERENCES