Journal of Healthcare and Advanced Nursing

ScitechJournals

Open Access

Insights into the Isolation and Identification of Medically Important Bacteria from Sputum Samples: A Comprehensive Review

Abstract

The isolation of medically important bacteria from sputum samples is a crucial process in diagnosing respiratory infections. This research paper investigates the potential pathogens in sputum samples obtained from patients with suspected respiratory tract infections. The study will employ various isolation and identification techniques, including culture-based methods and molecular techniques, to isolate and characterise the bacteria. Understanding the microbial composition of sputum samples can provide valuable insights into the etiology of respiratory infections and contribute to the development of more effective diagnostic and treatment strategies.

Keywords

Public health • Sputum samples • Bacterial isolation • Diagnostic methods • Microbiological analysis

Literature Review

Madhubala[1*], Amit Kumar Singh[2]

[1]Department of Basic Sciences, Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

[2]Department of Civil, Building and Environmental Engineering, Sapienza University of Rome, Italy

***Correspondence:** *Madhubala, Department of Basic Sciences, Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India, Email: madhubalachaudhary29@gmail.com*

Received: 22 August 2024; **Accepted:** 23 October 2024; **Published:** 07 November 2024

Introduction

Isolating medically important bacteria from sputum samples is crucial in diagnosing respiratory infections. By identifying the specific bacteria present, healthcare professionals can determine the most effective treatment for patients. In this study, we aim to isolate and identify the medically important bacteria from the sputum sample collected from the local hospital of Hazartganj, Lucknow. Understanding the microbial composition of the sputum can provide valuable insights into the prevalence of respiratory pathogens in the region and contribute to improved management of pulmonary infections. Sputum, which is the mucus produced by the respiratory system, is a common specimen used for diagnosing pulmonary infections caused by medically important bacteria such as Mycobacterium tuberculosis complex and non-tuberculous mycobacteria. However, obtaining sputum samples can be challenging, especially in certain patient populations such as children and those with difficulty producing expectorated sputum. Moreover, young children cannot produce sputum; estimates indicate that they constitute about 10% of all new cases in high-burden areas. Furthermore, the traditional culture-based methods used for bacterial isolation from sputum samples can be time-consuming and labour-intensive. Additionally, these methods may require specialized equipment and reagents, making them costly and inaccessible in resource-limited settings. To overcome these challenges, our study will employ a modified protocol for isolating medically important bacteria from sputum samples.

Materials and Methods

In this study, we focused on the isolation of medically important microbes from sputum samples collected aseptically from the local hospital of Hazartganj, Lucknow. Nutrient Agar (NA) was prepared following a specific protocol. The components of NA included peptone, beef/yeast extract, sodium chloride (NaCl) and agar. The protocol for media preparation involved weighing all the reagents in a conical flask, followed by adding sterile water and heating to dissolve the constituents. After autoclaving at 121°C and 15 psi pressure for 15 minutes, the media were allowed to cool. Approximately 10 ml of nutrient agar was then added to each plate, which was swirled gently

to ensure coverage of the bottom. For spreading, sterile swabs were moistened in sterile water and the sputum samples were collected and spread onto NA plates. The inoculated agar plates were then incubated at 37°C in an inverted position for 24-48 hours. After 24 hours, mucoid colonies were observed and the plates were stored at 4°C for further identification. This standardized procedure ensured the successful isolation of medically important microbes from sputum samples, laying the foundation for subsequent microbial characterization and analysis.

Microscopic identification of bacteria isolated from sputum sample

Gram staining: We utilized the Gram staining technique to microscopically identify bacteria isolated from sputum samples. Developed by Dr. Christian Gram in 1884, Gram staining is a fundamental and widely used microbiological differential staining technique. It categorizes bacteria into Gram-positive or Gram-negative based on their cell wall structure, while also providing information on cell morphology, size and arrangement. This technique serves as the initial differential test conducted on specimens brought into the laboratory for identification, enabling rapid presumptive identification or elimination of particular organisms. The principle of Gram staining lies in the ability of bacteria to retain or lose the primary stain (crystal violet) when treated with a mordant (Gram iodine) and decolourizer (ethyl alcohol). Gram-positive bacteria retain the primary stain, appearing purple, while Gram-negative bacteria lose the stain and are counterstained with safranin, appearing pink. The procedure involves several steps, including preparation of a bacterial smear on a glass slide, staining with crystal violet and Gram iodine, decolorization with ethyl alcohol, counterstaining with safranin and observation under a light microscope.Upon microscopic examination of the stained smear, the observed bacteria were identified as Gram-positive cocci. This standardized Gram staining protocol provided valuable insights into the morphology and classification of the bacteria isolated from the sputum samples, contributing to their accurate identification and further characterization in our study.

Capsule staining: Capsule staining is a differential staining technique used to visualize the presence of capsules surrounding bacterial cells. Capsules are synthesized within the cytoplasm and secreted outside the cell, forming a protective layer around the bacterium. While most capsules are composed of a polysaccharide layer, some bacteria may have capsules made of polypeptides or glycoproteins. In capsule staining, acidic and basic dyes are used to stain the background and bacterial cells, respectively, facilitating visualization of the capsule. The staining procedure involves using crystal violet as the primary stain, which imparts a purple colour to all parts of the cell. Unlike other staining methods, capsule staining does not involve a mordant. A 20% copper sulphate solution serves as both the decolorizing agent and counterstain. This solution removes the crystal violet from the capsule while leaving the cell stained purple. As a result, the capsule appears as a faint blue halo surrounding the purple-stained cell. After the staining process, observation under oil immersion microscopy reveals purple cells surrounded by a clear or faint blue halo against a transparent background, confirming the presence of capsules.

Endospore staining: Endospore staining, employing the Schaeffer-Fulton method, is a differential staining technique utilized to differentiate between vegetative cells and endospores. Malachite green serves as the primary stain for endospores, which, due to their resistance, are forced to accept the dye through the application of heat. Heat acts as a mordant, permeating the spore wall with malachite green. To decolorize the vegetative cells, water is applied. As the endospores resist de-staining, they retain the primary dye, while the vegetative cells lose the stain. Safranin, a counterstain or secondary stain, is then applied to stain the decolorized vegetative cells. The procedure involves preparing smears of the organisms on a clean microscope slide, heat-fixing the smear and then applying malachite green. Heat is gently applied to the slide to allow the dye to permeate the spores. After cooling, the slide is rinsed with tap water to remove excess dye. Subsequently, the smear is stained with safranin, rinsed and air-dried. Observation under oil immersion microscopy reveals pink/red-coloured vegetative cells and the absence of spores after endospore staining.

Screening of microbes on selective media

Eosin Methylene Blue Agar: Screening microbes on selective media is crucial for identifying and differentiating

bacterial species based on their growth characteristics and metabolic abilities. Eosin Methylene Blue (EMB) agar and Mannitol Salt Agar (MSA) are two commonly used selective media in microbiology. EMB agar, containing peptone, lactose, sucrose and dyes eosin and methylene blue, is designed to distinguish between lactose fermenters and non-fermenters. Escherichia coli colonies on EMB agar exhibit a characteristic greenish metallic sheen, aiding in their identification.

Mannitol Salt Agar Media: On the other hand, MSA, composed of peptone, beef extract, sodium chloride, D-mannitol and phenol red, is used to differentiate staphylococci based on their ability to ferment mannitol. MSA appears in a red or orange colour and if bacteria hydrolyse mannitol, a yellow zone appears around the colonies. The streaking technique involves inoculating the media with bacterial colonies and incubating them for 24 hours. After incubation, observations on EMB agar revealed pink colonies indicative of lactose fermentation, while MSA plates showed no yellow zones, suggesting the inability of bacteria to hydrolyze mannitol. These selective media and techniques play a vital role in microbial screening and identification in laboratory settings.

Biochemical Characterization of Microbes

Methyl Red Test: Biochemical characterization of microbes involves several tests to understand their metabolic capabilities and identify specific traits. One such test is the Methyl Red Test, which assesses the ability of bacteria to utilize glucose and produce stable acids like lactic acid, acetic acid, or formic acid. In this test, bacteria are grown in MR broth containing glucose. If glucose is metabolized to produce a stable acid, the pH of the medium decreases to 4.5 or below, causing a change in the colour of the methyl red indicator from yellow to red. Following incubation, the addition of methyl red directly into the culture tubes reveals a red colour, indicating a positive test result.

Motility Test: Another important test is the Motility Test, which evaluates the ability of microorganisms to move independently. Motility is a characteristic feature of many bacteria, facilitated by flagella or other locomotor organelles. In this test, soft nutrient agar media is inoculated with the bacterial sample and incubated. The presence of haziness in the media indicates motility, as it suggests movement of the bacteria within the agar. Non-motile bacteria show no such haziness, indicating the absence of movement.

Carbohydrate Fermentation test: Carbohydrate Fermentation tests assess the ability of microorganisms to ferment carbohydrates, producing acids and gases as end products. This test involves inoculating glucose broths with bacteria and observing colour changes indicating pH alterations due to acid production. A change from red to yellow indicates acid production, suggesting positive fermentation. These biochemical tests provide valuable insights into the metabolic properties of microbes, aiding in their identification and classification.

Catalase Test: The Catalase Test serves as a diagnostic tool to identify bacteria capable of producing the enzyme catalase, which catalyses the breakdown of hydrogen peroxide into oxygen and water. This test is crucial for bacterial isolates less than 24 hours old. In the procedure, a small amount of bacteria from a 24-hour-old culture is placed on a microscopic slide, followed by the addition of hydrogen peroxide. The rapid formation of bubbles indicates a positive catalase test, suggesting the presence of the catalase enzyme. This enzyme enables bacteria to protect themselves from the harmful effects of hydrogen peroxide, a by-product of aerobic carbohydrate metabolism.

Lacto Phenol Cotton Blue (LPCB) staining: On the other hand, the isolation of fungi from different sources involves several steps, including culturing on Potato Dextrose Agar (PDA) media. PDA media, comprising potato infusion, dextrose and agar, provide a suitable environment for fungal growth. Additionally, the Lacto Phenol Cotton Blue (LPCB) staining method is commonly used for observing fungi. This technique involves staining with a solution containing phenol, cotton blue, glycerol and lactic acid, followed by microscopic examination. The resulting stained fungus can be identified based on its morphology and characteristics. Overall, these techniques contribute to the understanding of microbial diversity and behaviour, aiding in various research and diagnostic endeavours.

Synergistic effect of antibiotics by well diffusion method: The synergistic effect of antibiotics was evaluated using the well diffusion method. Initially, 15 ml of Nutrient Agar Medium (NAM) for bacteria was prepared and autoclaved, followed by cooling to approximately 45-

50°C. The freshly prepared medium was then poured into sterile glass or plastic petri plates and allowed to cool to room temperature. Subsequently, 30 µL of bacteria from a freshly prepared culture was pipetted onto the center of each petri plate and spread evenly across the surface using a spreader, followed by air-drying for 4-5 minutes. Three wells, each 6mm in diameter, were then created in the agar plates using a sterile borer. One well was loaded with 10 µL of ciprofloxacin, another with 10 µL of amoxicillin and the third with an equal amount of both antibiotics (10 µL each). The plates were then incubated at 37°C for 24 hours to allow for bacterial growth. Finally, the diameter of the zone of inhibition around each well was measured to assess the antibacterial activity of the antibiotics individually and in combination.

The antibacterial activity of different antibiotics was assessed by measuring the diameter of the zone of inhibition (mm). Amoxicillin exhibited a zone of inhibition measuring 2.1 mm, while ciprofloxacin displayed a slightly larger zone of inhibition at 2.3 mm. Interestingly, when amoxicillin and ciprofloxacin were combined, the zone of inhibition increased to 2.6 mm, suggesting a synergistic effect between the two antibiotics. This observation underscores the potential for combination therapy to enhance the efficacy of antibiotic treatment against bacterial infections.

Results

After incubation, mucoid colonies were observed on the agar plates, indicating successful growth of the cultured samples, which were subsequently stored at a controlled temperature of 40°C to facilitate further characterization. Subsequent Gram staining of the bacterial cultures revealed them to be gram-positive cocci, providing initial insights into their morphological characteristics. **(Figure1)** Capsule staining elucidated the presence of capsules surrounding the bacterial cells, evidenced by the visualization of purple cells encased in a clear or faint blue halo against a transparent background. Endospore staining confirmed the absence of endospores, with vegetative cells displaying a pink/red hue. Further analysis of selective media, including EMB and MSA, revealed specific growth patterns. Pink colonies observed on the EMB media suggested lactose fermentation, while the absence of light-yellow zones on the MSA media indicated the inability of the bacteria to hydrolyze mannitol. **(Figure 2)** Biochemical tests, including the methyl red test, demonstrated the metabolic capabilities of the bacterial isolates, with the formation of a distinct red ring in the culture broth indicating positive results. Additionally, observations of carbohydrate fermentation and bubble formation upon exposure to H2O2 further characterized the microbial strains. Microscopic examination of stained fungal samples revealed morphological features resembling Rhizopus, contributing to the broader understanding of microbial diversity and behaviour within the studied samples. These findings collectively enhance our understanding of the physiological and biochemical attributes of the tested microbial strains, offering valuable insights into their potential roles in various environmental and clinical settings.

Figure 1

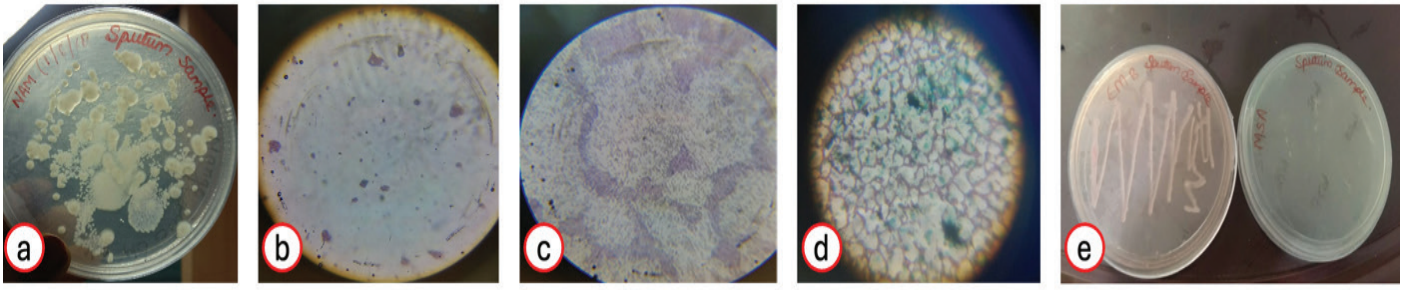

Figure 1A. Bacterial colonies isolated from sputum samples were observed after 24 hours of incubation.
Figure 1B. Following bacterial staining, the organisms exhibited a Gram-positive morphology and appeared in cocci shapes.
Figure 1C. The presence of capsules was confirmed by the observation of purple cells surrounded by a clear or faint blue halo against a transparent background.
Figure 1D. Vegetative cells exhibited a pink/red colouration, while spores were notably absent as indicated by the lack of green colouration.

Figure 1E. Growth was observed on the EMB plate, displaying a pink hue, whereas no growth was evident on the MSA plate.\

Figure 2

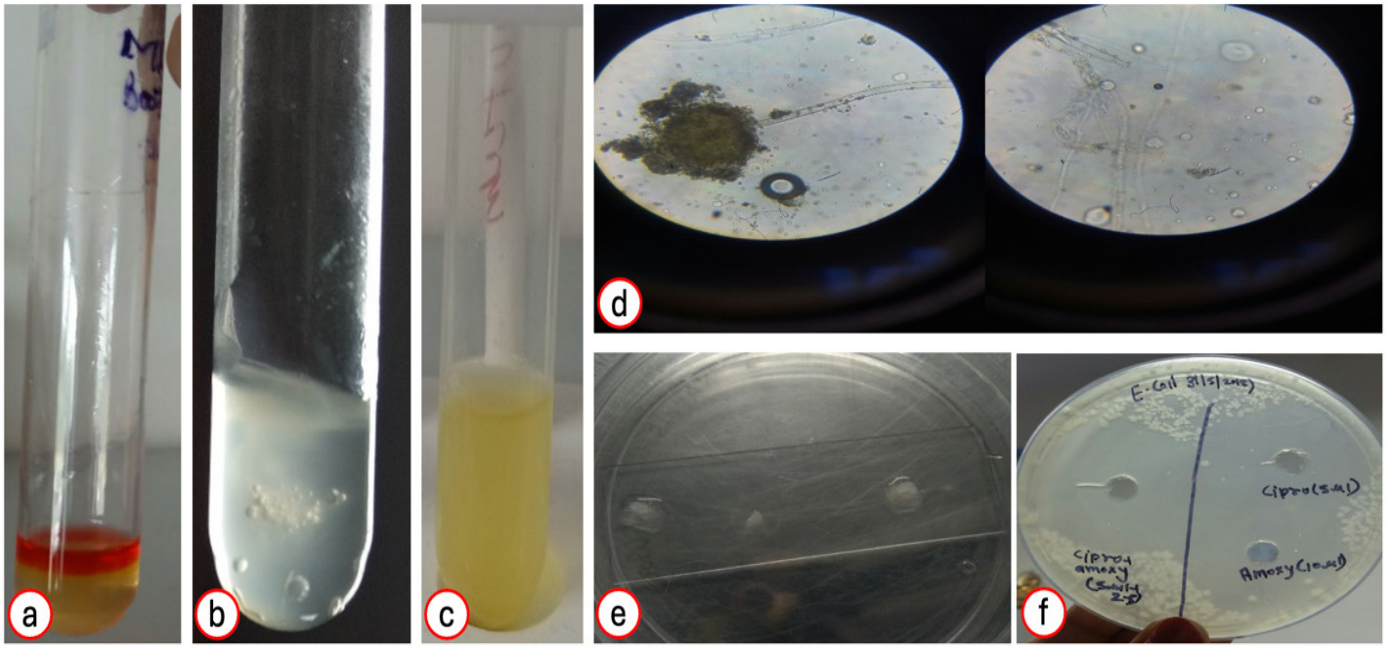

Figure 2A. The appearance of a red ring on the media was noted after the addition of methyl red, indicating a positive test result.
Figure 2B. Observation revealed non-motile bacterial behavior; no motility was detected on semi-solid media.
Figure 2C. A distinct colour change from red to yellow was observed on the media.
Figure 2D. Positive test results were confirmed with the formation of bubbles.
Figure 2E. Fungus specimens were subjected to staining using lactophenol cotton blue.
Figure 2F. Zones of inhibition against E. coli were observed.

Conclusion

In conclusion, the various staining techniques and selective media discussed in this document play a crucial role in microbial screening, identification and characterization. These essential microbiological techniques provide valuable insights into the metabolic properties, structure and behavior of different microorganisms. Additionally, biochemical tests such as the Methyl Red Test, Motility Test, Carbohydrate Fermentation tests and Catalase Test offer significant information about the metabolic capabilities and specific traits of bacteria, facilitating their identification and classification. Moreover, the isolation and observation techniques for fungi, including culturing on Potato Dextrose Agar media and staining with Lacto Phenol Cotton Blue, contribute to the understanding of microbial diversity and aid in various research and diagnostic endeavors. Overall, these techniques are indispensable for microbiologists in the laboratory setting and significantly contribute to microbiology and related research.

Acknowledgment

We would like to thank the Dr. Alka Saxena, CEO of the Acube Labs – Rapture Biotech, Lucknow, Uttar Pradesh, India for the technical and laboratory support.

References

1. *Amati, Francesco, Anna Stainer, Marco Mantero, Andrea Gramegna and Edoardo Simonetta, et al. "Lung microbiome in idiopathic pulmonary fibrosis and other interstitial lung diseases." Int J Mol Sci 23 (2022): 977.*

2. *Boehme, Catharina C., Mark P. Nicol, Pamela Nabeta and Joy S. Michael, et al. "Feasibility, diagnostic accuracy and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: A multicentre implementation study." The lancet 377 (2011): 1495-1505.*

3. *Drigot, Zoe G. and Sarah E. Clark. "Insights into the role of the respiratory tract microbiome in defense against bacterial pneumonia." Curr Opin Microbiol 77 (2024): 102428.*

4. *Garcia-Nuñez, Marian, Laura Millares, Xavier Pomares and Rafaela Ferrari, et al." J Clin Microbiol 52 (2014): 4217-4223.*

5. *Horn, Kadi J., Melissa A. Schopper, Zoe G. Drigot and Sarah E. Clark. "Airway Prevotella promote TLR2-dependent neutrophil activation and rapid clearance of Streptococcus pneumoniae from the lung." Nat Commun 13 (2022): 3321.*

6. *Lawn, Stephen D. and Robin Wood. "Tuberculosis in antiretroviral treatment services in resource-limited settings: addressing the challenges of screening and diagnosis." J Infect Dis 204, (2011): S1159-S1167.*

7. *Li, Zongjie, Yuhao Li, Qing Sun and Jianchao Wei, et al. "Targeting the pulmonary microbiota to fight against respiratory diseases." Cells 11 (2022): 916.*

8. *Neonakis, Ioannis K., Zoe Gitti, Elias Krambovitis and Demetrios A. Spandidos. "Molecular diagnostic tools in mycobacteriology." J Microbiol Methods 75 (2008): 1-11.*

9. *Palomino, J. C. "Nonconventional and new methods in the diagnosis of tuberculosis: Feasibility and applicability." (2005).*

10. *Palomino, Juan Carlos. "Molecular detection, identification and drug resistance detection in Mycobacterium tuberculosis." FEMS Immunol Med Microbiol 56 (2009): 103-111.*

11. *Parsons, Linda M., Ákos Somoskövi, Cristina Gutierrez and Evan Lee, et al. "Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities." Clin Microbiol Rev 24 (2011): 314-350.*

12. *Ramsheh, Mohammadali Yavari, Koirobi Haldar, Anna Esteve-Codina and Lillie F. Purser, et al. "Lung microbiome composition and bronchial epithelial gene expression in patients with COPD versus healthy individuals: a bacterial 16S rRNA gene sequencing and host transcriptomic analysis." Lancet Microbe 2 (2021): e300-e310.*

13. *Silva, Fabiola, Raphaël Enaud, Elizabeth Creissen and Marcela Henao-Tamayo, et al. "Mouse subcutaneous BCG vaccination and mycobacterium tuberculosis infection alter the lung and gut microbiota." Microbiol Spectr 10 (2022): e01693-21.*

14. *Somoskovi, Akos, Cristina M. Gutierrez and Max Salfinger. "Laboratory diagnosis of tuberculosis: novel and nonconventional methods." REV RES MED MICROBIO 19 (2008): 19-38.*

15. *Steingart, Karen R., Hojoon Sohn, Ian Schiller and Lorie A. Kloda, et al." Cochrane Database Syst Rev 1 (2013).*

16. *Wang, Zhendong, Chen Bai, Tingyao Hu and Changyong Luo, et al. "Emerging trends and hotspot in gut–lung axis research from 2011 to 2021: A bibliometrics analysis." Biomed Eng 21 (2022): 27.*

17. *Xiang, Li and Xianli Meng. "Emerging cellular and molecular interactions between the lung microbiota and lung diseases." Crit Rev Microbiol 48 (2022): 577-610.*

18. *GELMEZ, BÜŞRA, Ayca Yildiz-Pekoz and NİLÜFER YÜKSEL. "Lung Microbiota: Its Relationship to Respiratory System Diseases and Approaches for Lung-Targeted Probiotic Bacteria Delivery." (2023).*

Citation: Madhubala and Amit Kumar Singh. "Insights into the Isolation and Identification of Medically Important Bacteria from Sputum Samples: A Comprehensive Review." J Healthc Adv Nur (2024): 119. DOI: 10.59462/JHAN.2.3.119