# New technique use to Rapid Isolation Pneumococcus bacteria from both Pneumococcus meningitis and Pneumococcus pneumonia infections in Iraq

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**الخلاصة**

مرض ذات الرئة من الامراض الشائعة التي تصيب كافة الشباب وكبار السن وكذلك تسبب التهابات السحايا الدماغية للبالغين وتزداد الخطورة المتائتية نتيجة تعاطي الخمور وكذلك للذين يعانون من اصابات مزمنة كالتهابات الاذن الوسطى والجيوب الانفية وغيرها.

لغرض مقارنة نسبة الكفاءة للحصول على عزلات سريعة عند استخدام ثلاثة أوساط زرعية لعزل تلك البكتريا من المرضى المصابين بالتهابات السحايا الدماغية والالتهابات الرئوية فقد تم زرع مائة نموذج من السائل الشوكي ومن افرازات الجهاز التنفسي السفلي وقد لوحظ أن نسبة النمو على أوساط زرعية تحتوي على 5% من دم الخراف وكذلك في أوساط زرعية اخرى تحتوي على 5% دم الخراف مع إضافة 5 مايكروغرام من مضاد الحياة الجنتامايسين، وتم وضعها في الحاضنة في جو هوائي يحتوي على 5% من غاز ثنائي أوكسيد الكاربون حيث تكون نسبة النمو لا تتجاوز 70% في حين عند نمو نفس تلك الاوساط في جو لا هوائي تصل نسبة النمو لاكثر من90%.

**Abstract**

Pneumococcal pneumonia is more common in the very young and the very old ages. Pneumococci are the most common cause of meningitis in adults. Especially at risk are alcoholics and people with chronic otitis, sinusitis, mastoiditis. We compared the relative efficacy of three methods for the isolation of Streptococcus pneumoniae microorganism in both pneumococcal meningitis and pneumococcal pneumonia in both lower respiratory secretion and cerebrospinal fluid depending on results from total speciemens collected (Cerebrospinal fluid and respiratory secretion).

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These 100 speciemens were subcultured on different three types of blood agar plates we found that Streptococcus pneumonia was isolated at a frequency of 70% with 5% sheep Blood Agar or 5% Sheep Blood Agar containing 5ug/ml of Gentamicin per milliliter, both incubated in 5% $Co_2$. Anaerobic incubation of 5% sheep blood agar containing 5ug/ml of Gentamicin per milliliter enhanced the recovery rate of Streptococcus pneumonia to 90%. The differences in the recovery rates of Streptococcus pneumoniae isolated from both pneumococcal meningitis and pneumococcal pneumonia by various culture media and conditions were analyzed.
Keyword: Rapid Isolation of pneumococci in Different Media

## Introduction

Streptococcus pneumoniae is the most common cause of acute bacterial meningitis in adults, and even with appropriate antimicrobial therapy, case-fatality rates for high-risk patients can be as high as 55% for meningitis (1,2). An alarming increase of isolates with antimicrobial resistance has been noted (3-5) leading to shifts in the empirical antibiotic therapy for pneumococcal infections (6,7).

Meningococcal meningitis occurs most often during the first year of life. It also tends to occur in epidemics among closed populations (eg, in military barracks, college dormitories, and boarding schools) Pneumococci are the most common cause of meningitis in adults, especially at risk are alcoholics and people with chronic otitis, sinusitis, mastoiditis, CSF leaks, recurrent meningitis, pneumococcal pneumonia, sickle cell disease, or asplenia.

Incidence of pneumococcal meningitis is decreasing because of routine vaccination Pneumococcal pneumonia is more common in the very young and the very old. S. pneumoniae can be differentiated from Streptococcus viridans, some of which are also alpha-hemolytic, using an optochin test, as S. pneumoniae is optochin-sensitive. S. pneumoniae can also be distinguished based on its sensitivity to lysis by bile (the so-called "bile solubility test.").

The encapsulated, Gram-positive coccoid bacteria have a distinctive morphology on Gram stain, the so-called, "lancet-shaped" diplococci. They have a polysaccharide capsule that acts as a virulence factor for the organism; more than 90 different serotypes are known, and these types differ in virulence, prevalence, and extent of drug resistance. The laboratory diagnosis of invasive pneumococcal disease (IPD) continues to rely on culture-based methods that have been used for many decades (3-5).

The most significant recent developments have occurred with antigen detection assays, whereas the role of nucleic acid amplification tests has yet to be fully clarified. Despite developments in laboratory diagnostics, a microbiological diagnosis is still not made in most cases of IPD, particularly for pneumococcal pneumonia. The limitations of existing diagnostic tests impact the ability to obtain accurate IPD burden data and to assess the effectiveness of control measures, such as vaccination, in addition to the ability to diagnose IPD in individual patients. There is an urgent need for improved diagnostic tests for pneumococcal disease especially tests that are suitable for use in underresourced countries. Streptococcus pneumoniae (the pneumococcus) is one of the most important human pathogens. It is a major cause of pneumonia, meningitis, bacteremia, sinusitis, and otitis media, and it occasionally infects tissues at other sites. The collective term invasive pneumococcal disease (IPD) refers to pneumonia, meningitis, bacteremia, and infections of other normally sterile sites with The World Health (3-5).

The laboratory diagnosis of IPD currently relies on methods (or variations of methods) that have been around for many decades. The isolation rate of Streptococcus pnemoniae in sputum and cerebrospinal fluid cultures from patients with pnemococcal pneumonia and pneumococcal meningitis by the standard method, i,e, 5% sheep blood agar incubated in 5% $CO_2$ is low. Several studies have shown that approximately 60% of patients with bacteremic pneumococcal pneumonia yield positive culture of Streptococcus pneumoniae from the sputum(8,9) cerebrospinal fluid(CSF) (10) and 25% in sputum and CSF(10,11). This low

yield cannot be attributed to a decreased viability of the organism in the presence of pharyngeal flora and CSF when plating of the specimen delayed(12). The recognition of Streptococcus pneumoniae is hindered by the overgrowth of other less fastidious pharyngeal organisms when non selective media are used. The significant improvements in the recovery of Streptococcus pneumoniae with 5% sheep blood agar containing 5ug of gentamicin per milliliter is less efficient than the standard method for the isolation of Streptococcus pneumoniae.

Howden (1976) (13) has compared the results of aerobic and anaerobic cultivation for the primary isolation of Streptococcus pneumonia from the respiratory tract of children. His results indicated that about half of Streptococcus pneumoniae grew both aerobically and anaerobically; the remaining half grew only anaerobically. Since $CO_2$ was present in the anaerobic incubation only, it is not clear whether the improved recovery of Streptococcus pneumonia was due to the presence of $CO_2$, the anaerobic environment or both. An improved recovery rate of Streptococcus pneumoniae with anaerobic culture from the sputum of bacteremic pneumococcal pneumonia patients. The present study describes the results of a comparative of the yield of Streptococcus pneumonia from clinical specimens with various media and culture conditions. Our results indicate that anaerobic culture with sheep blood agar is the simplest procedure with the highest recovery of Streptococcus pneumoniae.

**Materials and Methods**

During a four months period, from June to October 2010 CSF and all sputa, bronchial washings and transtracheal aspirates were submitted for routine culture to the clinical laboratory of the Medical city hospital, Baghdad-Iraq were included in the study .Specimens were first screened microscopically for the suitability for culture. Acceptable specimens were processed for culture by the conventional protocol suggested by Isenberg et. al (14). Samples were collected for laboratory identification. Pneumococci are typically gram-positive, cocci, seen in pairs or chains. When

cultured on blood agar plates with added optochin antibiotic disk, they show alpha-hemolytic colonies and a clear zone of inhibition around the disk meaning they're sensitive to the antibiotic. Pneumococci are also bile soluble. Just like other streptococci, they are catalase-negative. A Quellung test can identify specific capsular polysaccharides. In addition to the standard culture media, additional plates of 5% sheep blood agar and gentamicin-containing sheep blood agar were used for each specimen. Throughout the study, all platting were carried out by the same technician and all media were prepared weekly so that uniform depth (6mm) was maintained for each batch of plates, gentamicin was supplied by Schering Drug company. The culture methods used in the present study are designated as follows: SBA-$CO_2$ for sheep blood agar incubated in 5% $CO_2$GBA-$CO_2$ for sheep blood agar supplemented with 5ug of gentamicin per milliliter incubated in 5% $CO_2$SBA-ANA for sheep blood agar incubated in a Gaspak jar (BBL, Microbiology, Systems, Cockeysville) After overnight incubations (18hours) at 37$^{o}$C, the plates were examined by three technicians working independently. The GBA-$CO_2$ plates were re-examined at the end of 48 hours of incubation.

Initial identification of Streptococcus pneumoniae was based on the typical colonial morphology seen under microscope. Each suspect was further checked by the bile solubility and Optician disk sensitivity rests of subcultures. In addition representatives of each morphological type of alpha-hemolytic colonies which did not resemble those of typical pneumococci were indiscriminately subcultures were carried out on 5% sheep blood agar incubated in 5% $CO_2$. Culture media giving no growth of Streptococcus pneumonia were reinsulated, using colonies from plates which yielded positive results. Quantization of bacterial growth based on the results of standard streaking on the original plates was scored as follows:

1 - (+) light growth in the primary streaking zone only
2 - (++) heavy growth in the primary streaking zone only
3 - (+++) growth in the primary and secondary streaking zone
4 - (++++) growth in the primary, secondary and tertiary streaking zone

The differences in the recovery rates of Streptococcus pneumoniae by various culture media and conditions were analyzed by using Yates correction of McNemar's test for significance (15). Of 100 acceptable specimens, there were 75 expectorated sputa, 20 bronchial washing and 5 transtrachael aspirates. Sixty specimens yielded Streptococcus pneumoniae by one or more techniques.And there 25 CSF specimens .Twenty two specimens yielded Streptococcus pneuminae by SBA-ANA. They yield of positive results by either SBA-$Co_2$ or GBA-$Co_2$ was similar (60 of 75 specimens 80%); SBA-ANA gave the best yield (70 of 75 specimens 93%).

**Results**

Of (100) acceptable specimens, there were (75) expectorated sputa, (20) bronchial washing and (5) transtrachael aspirates. Sixty specimens yielded Streptococcus pneumoniae by one or more techniques.And there (25) CSF specimens. Twenty two specimens yielded Streptococcus pneuminae by SBA-ANA. They yield of positive results by either SBA-$Co_2$ or GBA-$Co_2$ was similar (60 of 75 specimens 80%); SBA-ANA gave the best yield (70 of 75 specimens 93%). Although there are no significant differences between the results of SBA-$C0_2$ and GBA-$Co_2$, the differences between SBA-ANA and GBA-$Co_2$ and between SBA-ANA and SBA-$CO_2$ are highly significant (table 1). All isolates of Streptococcus pneumoniae were positive by the bile solubility test and produced a zone of growth inhibition of-15mm with the Optician disk sensitivity test plates. The colonies of Streptococcus pneumoniae on the SBA-$Co_2$ and GBA-$Co_2$, plates were quite similar in morphology, but different in size. Colonies were umbilicated with a central depression and non-mucoid in most isolates. Zones of alpha hemolysis surrounding these colonies were characteristically large. Because of the small size of colonies on GBA-$CO_2$ in about 2/3 of the cases the recognition of colony morphology characteristics of Streptococcus pneumoniae was possible only after 48 h of incubation. In contrast to the umbilicated appearance on both SBA-$CO_2$ and GBA-$CO_2$, the

colonies on SBA-ANA were domed in shape and the green hemolysis was virtually absent. A narrow green hemolysis became visible after a 30 minutes exposure of the plates in the air at room temperature. SBA-ANA colonies were consistently two to five times larger than their counterparts on SBA-$CO_2$ or GBA-$CO_2$ plates and had a characteristic grey, watery or mucoid appearance .Whereas most isolates were correctly recognized by their characteristic morphologies, the identification of Streptococcus pneumoniae in a few instances was a accomplished by indiscriminate subculturing of total positive results 6 to 8% by any of these culture techniques were obtained by sub culturing alpha-hemolytic colonies without the typical pneumococcal morphology. All 75 sputa and 25 CSF isolates grew well by the methods of SBA-$CO_2$, GBA-$CO_2$ and SBA-ANA on subculturing despite the fact that some of the isolates failed to grow on all plates initially.Positive specimens (103) yielded pure or nearly pure cultures of Streptococcus pneumoniae varying between 1+ to 4+ in quantity, whereas the concomitant pharyngeal organisms were present in quantities of 1+ or less. The remaining (22) positive specimens consisted of a mixture of 2+ to 4+ growth of pharyngeal organisms and a 1+to 4+ growth of Streptococcus pneumoniae .

The most common type of bacteria was viridians group streptococci followed by Staphylococcus aureus. Gram-negative enteric were found in only 4 cases (Table-2). To understand the mechanisms that explain the differences in the recovery rates and colony morphologies among the three methods, we did the following experiments. We compared the plating efficiencies of pure cultures from five clinical isolates of Streptococcus pneumoniae by the three methods. Result indicates that SBA-CO2, GBA-CO2 and SBA-ANA give essentially the same plating efficiencies in regard to the growth of Streptococcus pneumoniae. The ratio of average diameters of the colonies was 0.5 to 0.8; 1.2 to 5 (GBA-$CO_2$, SBA-$CO_2$, SBA-ANA). It was suggested by Howden (l976) (16) that the large mucoid colonies produced in the absence of oxygen were probably the result of the suppressed autolytic activity of Streptococcus pneuminae. Ontrary to previous reports,

the addition of gentamicin to sheep blood agar did not enhance the isolation rate of Streptococcus pneumonia in the lower respiratory secretions. The most striking effect of gentamicin in sheep blood agar is to eliminate gentamicin-susceptable gram-negative enteric and Staphylococcus aureus in the pharyngeal flora. Other species such as viridians group streptococci, diphthroids, neisseria species and yeasts are not affected by the presence of these antibiotics. Of 100 clinical specimens, 13% of the positive specimens had significant number of Staphylococcus aureus and negative enteric. The smaller colonies of Streptococcus pneumonia on GBA-$CO_2$ plates presented difficulties in distinguishing Streptococcus pneumoniae from other alpha-hemolytic streptococci even after 48 h of incubation, this difficulty could account for all (24) false-negative results. In one specimen the presence of viridians group streptococci prevented the recognition of Streptococcus pneumonia inspires of the complete elimination of Escherichia coli on the GBA-$CO_2$ plate. In view of the nature of colonized organism in our patient populations and the difficulty with the recognition of Streptococcus pneumoniae, we believe GBA-$CO_2$ is of limited value for the primary culturing of Streptococcus pneumonia from lower respiratory tract secretion SBA-ANA enhanced the isolation rate of Streptococcus pneumoniae by 50% as compared with the standard method. There were four-false negative speciemens. In one case, the failure to recognize Streptococcus pneumoniae can be attributed to the growth of a large quantity of mucoid Klebsiella pneumoniae. The remaining three were due to the difficulty in distinguishing Streptococcus pneumoniae from other oral streptococci. In the majority of cases the colonies of Streptococcus pneumoniae could be easily recognized by their larger sizes and mucoid appearance in the midst of normal flora whereas organism such as Staphylococcus aureus, gram-negative enteric, diphthroids, Neisseria species and yeast were suppressed under anaerobic incubation (16) Strict anaerobes which are prevalent in the mouth and upper respiratory tract as normal flora, did not interfere in the SBA-ANA method. All 100 specimens were collected and handled without taking special precaution against oxygen toxicity. In

addition SBA-ANA plates were examined after 18 h of incubation period too short for most fastidious anaerobes to become visible on the plates.

In spite of the fact that all 125 isolates could grow both aerobically and anaerobically on subculturing , we cannot rule out the possibility that some strain of Streptococcus pneumonia may be non-aerotolerant during initial isolation. Since our initial screening focused on alpha-hemolytic colonies, it is likely that the beta-hemolytic obligatory anaerobic Streptococcus pneumoniae is currently classified as a facultative anaerobe

**Discussion**

After overnight incubation at 35°C with 5% $CO_2$ on 5% sheep blood agar or chocolate agar, S.pneumoniae colonies appear to be small, grayish, and mucoid and are surrounded by a greenish zone of α-hemolysis. After 24–48 h of incubation, the colonies become centrally depressed ("draughtsman" colonies). Further identification is important to confirm the identity. Laboratory differentiation between S.pneumoniae and other viridans streptococci is usually accomplished by 2 key reactions: optochin susceptibility and bile solubility. Optochin (ethylhydrocupreine) is an antibacterial agent that is not used therapeutically but is used for the laboratory identification of streptococci. The bile solubility test is based on the autolysis of S.pneumoniae in the presence of the surfactant sodium deoxycholate. S.pneumoniae isolates are typically susceptible to optochin and are bile soluble, whereas other viridans streptococci are typically resistant to optochin and are bile insoluble. Although bile solubility is generally regarded as being very sensitive and specific for identification of, S.pneumoniae the finding that up to 10% of S.pneumoniae isolates can be resistant to optochin has reduced reliance on the latter test (17) consequently, suspicious isolates that have reduced susceptibility to optochin should also be tested for bile solubility.

In pneumococcal meningitis, documented bacteremia occurs more frequently than in pneumonia, and reported rates of positive results are often >50% .The relatively low documented rates of

bacteremia in patients with IPD involve several factors, including prior administration of antimicrobials and the intermittent nature of bloodstream invasion by S.pneumoniae. In addition, S.pneumoniae releases autolysin during the stationary growth phase, resulting in cell death and making traditional methods of bacterial growth on media, such as blood culture, difficult(18). CSF examination for the diagnosis of pneumococcal meningitis, the combination of Gram stain and bacterial culture of CSF samples will identify most cases. In one large review, Gram stain smears of CSF samples detected S.pneumoniae with a sensitivity of 84% and specificity of 98%, but prior administration of antibiotics significantly reduced the yield for both Gram stain smear and culture (18). Processing a CSF specimen for culture as soon as possible is vital for optimal culture performance, because bacterial viability decreases over time, sputum examination. In the absence of documented bacteremia, the diagnosis of pneumococcal pneumonia can be challenging, especially in children who may not produce sputum. The microscopic demonstration of numerous gram-positive diplococci in a sputum sample containing <10 squamous epithelial (SEC) cells and >25 polymorphonuclear (PMN) cells per low-power field (magnification, ×100)(19) or 10 leukocytes for each SEC (19) for a patient with pneumonia is strongly suggestive of pneumococcal pneumonia. This is further supported if S.pneumoniae is the predominant isolate in cultures of sputum specimens. Poor-quality sputum samples, which contain relatively low numbers of PMN cells and high numbers of SEC cells, should not be processed, because they are likely to represent commensal oropharyngeal flora. Having a sputum quality assessment system in place is a valuable and cost-effective tool that allows the microbiology laboratory to maintain clinically relevant results. In this context, it is useful to remember that intra- and intertechnologist variability in the specimen quality assessment process have been reported and might account for some of the varied sensitivities of Gram-stained sputum specimens for the detection of pneumococci(19,20).

Several clinical studies have shown that sputum culture and Gram stain are still useful for the diagnosis of pneumococcal pneumonia, as long as specimens are of high quality and, ideally, were obtained before the administration of antibiotic therapy or up to 24 h after the initiation of therapy (19,20). One prospective study revealed that high-quality sputum samples can be obtained from a substantial proportion of adults with community-acquired pneumonia and that the sputum Gram stain had sensitivity of 57% and specificity of 97% for the diagnosis of pneumococcal pneumonia (20). For bacteremic pneumococcal pneumonia in adults, sputum Gram stain and culture have sensitivities of 80% and 93%, respectively, if an adequate specimen has been produced before therapy (19). The reason why sputum culture has been shown to have variable sensitivities in different studies is not necessarily the inadequacy of the microbiological tool itself; it can be the result of various factors, such as delayed processing of a sputum sample or processing of an inadequate sample, the patient's failure to produce a sputum sample, and the administration of antimicrobial therapy before obtaining a specimen. This study does not intended to establish the etiological diagnosis in pneumococcal pneumonia patient, but to explore a simple cost-effective method to improve the rate of isolation of Streptococcus pneumonia in clinical laborotaries.

We conclude that SBA-ANA is simple procedure with better results than the standard method and gentamicin-supplemented blood agar.

**Table-1: Comparison of the number of specimens in different media gives positive culture of Streptococci A-SBA-Co2 verses GBA-Co2**

| Sample | No.of positive samples in each media cultivated | | | |
|---|---|---|---|---|
| | SBA-Co2 | Percentage | GBA-co2 | Percentage |
| Sputa | 60/75 | 80% | 60/75 | 80% |
| Broncheal washing | 14/20 | 70% | 15/20 | 75% |
| Transtreacheal | 2/5 | 40% | 2/5 | 40% |
| CSF | 14/25 | 56% | 16/25 | 64% |

**B- SBA-ANA verses SBA-Co2**

| Sample | No.of positive samples in each media cultivated | | | |
|---|---|---|---|---|
| | SBA-ANA | Percentage | SBA-co2 | Percentage |
| Sputa | 70/75 | 93% | 60/75 | 80% |
| Broncheal washing | 18/20 | 90% | 14/20 | 70% |
| Transtreacheal | 3/5 | 60% | 2/5 | 40% |
| CSF | 22/25 | 80% | 14/25 | 56% |

**C- SBA-ANA verses GBA-ANA**

| Sample | No.of positive samples in each media cultivated | | | |
|---|---|---|---|---|
| | SBA-ANA | Percentage | GBA-ANA | Percentage |
| Sputa | 70/75 | 93% | 60/75 | 80% |
| Broncheal washing | 18/20 | 90% | 14/20 | 70% |
| Transtreacheal | 3/5 | 60% | 3/5 | 60% |
| CSF | 22/25 | 80% | 16/25 | 64% |

**D-SBA-ANA verses GBA-Co2**

| Sample | No.of positive samples in each media cultivated | | | |
|---|---|---|---|---|
| | SBA-ANA | Percentage | GBA-C02 | Percentage |
| Sputa | 70/75 | 93% | 60/75 | 80% |
| Broncheal washing | 18/20 | 90% | 15/20 | 75% |
| Transtreacheal | 3/5 | 60% | 2/5 | 40% |
| CSF | 22/25 | 80% | 16/25 | 64% |

**Table-2; Concomitant pharyngeal organisms with 2+ to 4+ growth from 60 Streptococcus pneumonia positive specimens**

| Organisms | No. of Specimens |
|---|---|
| Diphthroid | 1 |
| Eschreschia coli | 1 |
| Klebsiella pneumonia | 4 |
| Neisseria species | 2 |
| Proteus mirabilis | 1 |
| Staphylococcus aureus | 6 |
| Viridans group streptococci | 11 |

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