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# Isolation, Identification, and Antimicrobial Susceptibility Testing of *Streptococcus pneumoniae*

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1 Works for me dx.doi.org/10.17504/protocols.io.bkuakwse

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ABSTRACT

*Streptococcus pneumoniae* (*S. pneumoniae*) is a major cause of bacterial pneumonia and meningitis globally. This bacterium is a normal flora of nasopharynx but can move to sterile sites such as cerebrospinal fluid, blood, and pleural fluid then causes invasive pneumococcal diseases. However, antibiotics resistance causes the failure of pneumococcal disease treatment. *S. pneumoniae* penicillin-resistant is one of the priority antimicrobial resistant (AMR) pathogens in WHO list. Since pneumococcus is a fastidious bacteria, isolation and identification of *S. pneumoniae* is challenging. This bacteria need good quality of blood agar plate to grow and proper method to identify *S. pneumoniae*. Performing antimicrobila susceptibility testing of *S. pneumoniae* isolates is important to monitor and handle its resistance. Therefore, we decided to write the protocol regarding isolation, identification, and antimicrobial susceptibility testing of *S. pneumoniae*.

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PROTOCOL CITATION

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KEYWORDS

Streptococcus pneumoniae, isolation, identification, antimicrobial



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41570

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| TSA II Trypticase Soy Agar Modified | 212305 | Becton-Dickinson |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| DD1 Optochin | X3483A | Oxoid Microbiology Products - Thermo Fischer |
| Sodium deoxycholate | SRE0046-100G | Sigma |
| Mueller-Hinton Agar | CM0337 | Oxoid Microbiology Products - Thermo Fischer |
| Tris-EDTA buffer solution | 933283 | Sigma – Aldrich |
| Skim Milk | 232100 | Becton-Dickinson |
| Tryptone Soya Broth | CM0129 | Oxoid Microbiology Products - Thermo Fischer |
| Dextrose | 215530 | Becton-Dickinson |
| Glycerol | G5516 | Sigma |
| McFarland Equivalence Turbidity Standard | R20421 | |
| Clindamycin | CT0064B | Oxoid Microbiology Products - Thermo Fischer |
| Erythromycin | CT0020B | Oxoid Microbiology Products - Thermo Fischer |
| Oxacillin | CT0159B | Oxoid Microbiology Products - Thermo Fischer |
| Chloramphenicol | CT0013B | Oxoid Microbiology Products - Thermo Fischer |
| Tetracycline | CT0054B | Oxoid Microbiology Products - Thermo Fischer |
| Sulphametoxazole/Trimethoprim | CT0052B | Oxoid Microbiology Products - Thermo Fischer |
| Gentamicin Solution | G1397 | Sigma |
| FLOQ Swab | 503CS01 | Copan |

## Nasopharyngeal Swab Collection

1 Nasopharyngeal swab collection follow method as described previously

> Satzke C, Turner P, Virolainen-Julkunen A, Adrian PV, Antonio M, Hare KM, et al. (2013). Standard method for detecting upper respiratory carriage of Streptococcus pneumoniae: Updated recommendations from the World Health Organization Pneumococcal Carriage Working Group. Vaccine. 2013 Dec;32(1):165–79.. http://dx.doi.org/10.1016/j.vaccine.2013.08.062

. A flexible nasopharyngeal flocked swab is slowly passed into nasopharynx. The swab should not be resistant before it was reached nasopharynx (NP). The nasopharynx distanced one-half to two-third from nostril to ear lobe

2 Once the swab reaches nasopharynx, it is rotated 3-5 times to let the mucus absorb into the swab

3 The swab is removed slowly and immadiately placed into 1 mL of Skim milk Tryptone Glucose and Glycerol (STGG). STGG **100 mL** was made by mixing **2 g** skim milk powder, **3 g** Tryptone Soya Broth, **0.5 g** glucose/dextrose, glycerol **10 mL** and distilled water **90 mL**

4 The NP-STGG is vortexed for 10-20 sec to disperse the bacteria from swab to the STGG then placed it in cryobox that



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filled by ice packs. The temperature should be **4 °C** and it is regularly monitored through the digital temperature controller

5 The inoculated STGG is kept at **4 °C** for 4-6 hours after collection until reaches targeted laboratory. After that, the swab-STGG is frozen at **-80 °C** to secure survival of pneumococcus

6 The specimens are shipped to Molecular Bacteriology Unit, Eijkman Institute using dry ice.

7 Once it is arrived Eijkman Institute, the swabs were immadiately stored at **-80 °C** until culture.

## Optochin Susceptibility Test

8 NP-STGG medium that stroed in **-80 °C** is thawed and vortexed to disperse the bacteria from the swab.

9 **20 µl** of of swab-inoculated STGG was transferred and streaked onto blood agar plate (TSA II Trypticase Soy Agar Modified) with **8 % (v/v)** sheep blood and supplemented with 5 mg/L gentamicin.

Hadinegoro SR, Prayitno A, Khoeri MM, Djelantik IGG, Dewi NE, Indriyani SAK, et al.. Nasopharyngeal Carriage of Streptococcus pneumoniae in Healthy Children Under Five Years Old in Central Lombok Regency, Indonesia.. Southeast Asian J Trop Med Public Health. 2016;47(3):9..

10 The plate is incubated at **37 °C** with 5% $CO_2$ for 18-24 hours **0 Mass Percent**

11 Single colony of alpha-hemolytic and flat-depressed colony resembling pneumococci is picked and streaked onto blood agar plate (TSA II Trypticase Soy Agar Modified) with **8 % (v/v)** sheep blood.

12 Quadran 1 and 2 is overlapped to test optochin susceptibility of the isolate. If there are 2 different colonies in the plate, each of them should be tested for optochin susceptibility separately.

13 Optochin disk (ethylhydrocupreine hydrochloride) is placed on the overlap area between quadran 1 and 2.



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14 Susceptible isolate (zone ≥14 mm) with distinct characteristics of pneumococci (flat depressed center, shiny or wet colonies), is defined as *Streptococcus pneumoniae* (*S. pneumoniae*)

15 Overnight culture is harvested and put into STGG then stored in **-80 °C**

Bile Solubility Test

16 Resistant optochin isolate (zone ≤ 14 mm) which has characteristics of S. pneumoniae or susceptible isolate which has no pneumococcus disticnt characteristics (too dry colonies), is contined to bile solubility testing

https://www.cdc.gov/meningitis/lab-manual/chpt08-id-characterization-streppneumo.pdf

17 For each isolate tested, two glass tubes are prepared and labeled as 'test tube' and 'control tube'

18 2 mL and 1 mL of saline 0.85% are added into test tube and control tube, respectively

19 Bacterial suspension that equvialent to MacFarland standard 1 is made in the test tube. Bacterial density is measued by densitometer (make sure to calibrate prior to using)

20 1 mL of bacterial suspension from test tube is transferred into control tube then vortexed.

21 **1 mL** of bile salt or sodium deoxycholate **2 Mass Percent** is added into test tube. The final volume of test tube and control tube are **2 mL** for each

22 ATCC 49619 of *S. pneumoniae* must be included in every bile testing

23 Test tube and control tube are incubated room temperature for 10 minutes. If the solution in the test tube of isolate is as clear as the test tube of ATCC 49619 (the soulution should be as clear as water), the isolate is bile-soluble and defined as S. pneumoniae. Otherwise, if there is no turbidity difference between test tube and control tube of the isolate, the isolate was not bile-soluble

24 If the isolate is negative for the first 10 minutes incubation, then continue for incubation at room temperature for 2 hours

25 Bile soluble isolate is streaked onto blood agar plate (TSA II Trypticase Soy Agar Modified) with **8 % (v/v)** sheep



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blood and incubated at **37 °C** with 5% $CO_2$ for 18-24 hours

26 Bile-soluble isolates were harvested and put into labeled STGG for freezing stock then vortexed. The bacteria should be well-hemogenized in STGG

27 STGG-inoculated isolate is stored in -80°C

## DNA Extraction of *S. pneumoniae*

28 The DNA extraction of *S. pneumoniae* is performed by boil method as described previously

Pai R, Gertz RE, Beall B. Sequential Multiplex PCR Approach for Determining Capsular Serotypes of Streptococcus pneumoniae Isolates. J Clin Microbiol. 2006;44:8..
http://10.1128/JCM.44.1.124–131.2006

Pure colonies of *S. pneumoniae* stored in STGG at-80°C are streaked onto 8% sheep blood agar plate. Inoculated STGG must be kept at **4 °C**

29 Incubation at **37 °C** for 18-24 h with 5% $CO_2$.

30 Single colony of the isolate is picked and streaked onto 8% sheep blood agar plate then incubated for 18-24 h at **37 °C** with 5% $CO_2$.

31 Fresh culture is harvested into TE buffer in **1.5 mL** microcentrifuge tube and vortexed until homogenized

32 Bacterial suspension is heated at **100 °C** for **00:05:00** and immadiately placed at freezer **-20 °C** **00:05:00**

33 Centrifuge at **13000 x g, 00:10:00** then kept at **-20 °C** until serotyping

## Serotyping of *S. pneumoniae*

34 Serotyping of *S. pneumoniae* was performed by sequential multiplex conventional PCR targeted *wzy* gene and consisted of 8 reactions



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Carvalho M d. G, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, et al. (2010). Revisiting Pneumococcal Carriage by Use of Broth Enrichment and PCR Techniques for Enhanced Detection of Carriage and Serotypes. J Clin Microbiol. 2010 May 1;48(5):1611–8. http://JOURNAL OF CLINICAL MICROBIOLOGY, May 2010, p. 1611–1618

. Latin America scheme as described by Centers for Disease Control and Prevention, is followed for serotyping the isolates **Latin America Scheme Multiplex PCR.pdf** . Every reaction should be detect *cpsA* gene. Each reaction has different serotypes to be identified **PCR Oligonucleotide Primers.pdf** .

Reaction 1: serotype14, 6A/6B/6C/6D, 23F, 19A, and 9V/9A.

Reaction 6C: serotype 6A/6B/6C/6D and 6C/6D.

Reaction 2: serotype 19F, 3, 15B/15C, 18C/18F/18B/18A, and 17F.

Reaction 3: serotype 1, 5, 9N/9L, 7F/7A, and 16F.

Reaction 4: serotype 8, 2, 4, 20, and 22F/22A.

Reaction 5: serotype 7C/7B/40, 12F/12A/44/46, 11A/11D, 10A, and 23A.

Reaction 6: serotype 21, 33F/33A/37, 15A/15F, 35F/47F, and 13.

Reaction 7: serotype 39, 23B, 35A/35C/42, 38/25F/25A and 35B.

Reaction 8: serotype 24F/24A/24B, 10F/10C/33C, 34, 19Fvar, and 31.

35 Mastermix for each reaction (25 µl) is prepared as described in Latin America scheme spreadsheet. For example, reaction 1 is performed by adding PCR $H_2O$ **0.5 µl** of 5× PCR buffer (Promega), **3.5 µl** of $MgCl_2$ 25 mM, **1 µl** of dNTPs (Promega) 5 mM, varied volume for 25 µM forward primer and 25 µM reverse primer, **0.2 µl** Taq Polymerase 2U and **2.5 µl** of DNA template, into 1.5 mL of microsentrifuge tube.

36 For isolates serotyping, conventional PCR is run under following condition: pre-denaturation at **94 °C** for **00:04:00** , followed by 30 cycles of denaturation at **94 °C** for **00:00:45** , annealing at **54 °C** for **00:00:45** , and extention at **65 °C** for **00:02:30** .

37 The DNA is visualized through electrophoresis. Agarose **2 Mass Percent** is made by adding 2 gram of agarose into 100 mL of Tris Acetat EDTA (TAE) 1× then heated in the microwave.



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38 Agarose is poured into electrophoresis tank with the comb.

39 Agarose gel is placed into electrophoresis tank filled with cold TAE 1×

40 PCR product and DNA ladder 50 bp are added into each well of agarose gel

41 Voltage is set to 100V and run for 90 minutes

42 DNA bands are visualized using GelDoc machine

43 Isolate that serotype-negative is confirmed by real-time PCR targeting *lytA* to confirm that the isolate was *S. pneumoniae*. For isolate with positive *lytA is* assigned as non-typeable (NT) by PCR *S. pneumoniae*

Carvalho M d. GS, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, et al (2007). Evaluation and Improvement of Real-Time PCR Assays Targeting lytA, ply, and psaA Genes for Detection of Pneumococcal DNA. J Clin Microbiol. 2007 Aug 1;45(8):2460–6.
http://10.1128/JCM.02498-06

## Antimicrobial Susceptibility Testing

44 Antimicrobial susceptibility testing is performed by following Clinical Laboratory Standard Institutes (CLSI) 2017 guidelines

Clinical and Laboratory Standards Institute. M100 Performance standards for antimicrobial susceptibility testing 27th Edition (2017).

. Frozen isolate in STGG was streaked onto [M] **8 % (v/v)** sheep blood agar plate. The STGG must be kept at **4 °C** condition.

45 Single colony of pneumococci is picked and cultured onto 8% sheep blood agar plate then incubated for 18-24h at 37°C with 5% $CO_2$.

46 Bacterial suspension is made by harvesting freshly grown *S. pneumoniae* and put into 5 mL saline



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[M]0.85 Mass Percent

47 The suspension is adjusted to be equivalent as McFarland standard 0.5. Densitometer is used to measure the turbidity. Calibration using McFarland standards is done prior to measurement.

48 When the suspension reaches 0.5 McFarland standard, sterile cotton swab is dipped into bacterial suspension then pressed gently on the wall of glass tube to remove excess fluid.

49 The cotton swab is lawned confluently onto Mueller Hinton Agar (MHA) plate with [M]**5 % (v/v)** sheep blood and streaked 3 times by rotating in 60° direction.

50 Antibiotic disks are placed onto the agar and pressed gently by sterile pinset.

Clindamycin 2 µg, chloramphenicol 30 µg, oxacillin 1 µg, tetracycline 30 µg, erythromycin 15 µg, and trimethophrim/sulfamethoxazole 1,25/23.75 µg, are used. Oxacillin disk is applied to measure susceptibility of isolates to penicillin.

51 Inoculated MHA is incubated at **37 °C** with 5% $CO_2$ for 20-24 h.

52 Inhibition zone is measured and recorded. Interpretation of clear zone follow Clinical Laboratory Standards Institute guideline 2017.



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