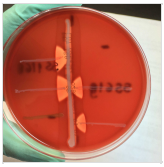

May 03, 2021

Group B Streptococcus (*Streptococcus agalactiae*) Isolation, Identification and Antimicrobial Susceptibility Testing

Wisnu Tafroji[1], Maria da Gloria Carvalho[2], Fabiana C Pimenta[2], Miftahuddin majid khoeri[1], Dodi Safari[1]

[1]Eijkman Institute for Molecular Biology, Jakarta, Indonesia;
[2]Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, United States

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Eijkman Institute for Molecular Biology

Wisnu Tafroji
Eijkman Institute for Molecular Biology, Jakarta, Indonesia

ABSTRACT

This protocol provides a guideline for isolation, identification and antimicrobial susceptibility testing by microdilution of Group B Streptococcus (GBS) / *Streptococcus agalactiae.* The isolation is done by incubation of swab specimens in LIM broth enriched with 1.0 mL of rabbit serum, followed by selective media chromoagar. The identification is performed according to biochemistry characteristics by observing CAMP activity. Definitive grouping was done by latex agglutination (Remel cat no. R62031), according to the manufacturer's instructions. The DNA was obtained using the fast extraction methodology for *Streptococcus*isolates and serotyping of GBS isolates was performed by conventional multiplex PCR. Antimicrobial susceptibility testing by broth microdilution was performed following the recommendations of Clinical and Laboratory Standards Institute minimum inhibitory concentration (MIC) breakpoints.

DOI

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KEYWORDS

GBS, Vaginal Swab, Isolation, Identification, Serotype, Antimicrobial resistance, Streptococcus agalactiae

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Broth enrichment V/R swab culture for enhanced GBS growth 7h

7h

1

1. Thaw the Vaginal/Rectal (V/R)-STGG specimens at room temperature (25^{O}C) and vortex for approximately 10-20 seconds.
2. Re-freeze the specimen (*i.e.*, the STGG) as soon as possible and keep it cool in an ice bath during processing.
3. Avoid multiple freeze-thaw cycles whenever possible.
4. Transfer 200 µl of the V/R-STGG to 5 ml of LIM broth (BD-BBL cat# 296266) containing 1 ml of rabbit serum (Gibco cat#16120099).
5. Vortex and incubate the inoculated LIM broth for 5 hours at 37^{O}C with 5% CO_2.
6. Vortex and inoculate one loop (10 µl) of the LIM broth enriched culture on CHROMID®*Strepto* B (BioMérieux cat#419751) and incubate for 18-24 hours at 37 ^{O}C in CO_2-incubator.

GBS isolates detection and identification 2d

2d

2

1. Carefully examine the CHROMID®Strepto B, for typical GBS colonies, pale pink to red after 24 hours incubation.
2. A single colony (suspected pink colony) should be passed to fresh blood agar plate (BAP) and incubate for 18-24 hours at 37^{O}C in CO_2-incubator. The GBS colonies are gray to whitish gray surrounded by a weak zone of beta hemolysis. Non-hemolytic isolates can also be encountered.
3. The suspected GBS isolates should be submitted to identification test, including Gram stain, CAMP test and latex agglutination (PathDx Strep B latex – R62031).
4. If confirmed identification all GBS isolates should be frozen from a fresh culture (24h) in STGG or sheep blood and kept at -70°C for further testing.

To perform the CAMP-test 2d

2d

3

1. The CAMP test (Christie-Atkins-Munch-Peterson) is a test to identify GBS based on their formation of a substance (CAMP factor) that enhanced the area of hemolysis formed by the beta-hemolysin elaborated from *Staphylococcus aureus* (ATCC 25923).
2. The suspected GBS strain to be tested and the *S. aureus* (ATCC 25923) are streaked onto a BAP at 90^{O} angle.
3. Incubate at 37°C/CO_2 for 18-24 hours. A positive reaction can be detected by the presence of a triangular zone of enhanced beta-hemolysis in the diffusion zone of *S. aureus* beta-hemolysin and the CAMP factor.

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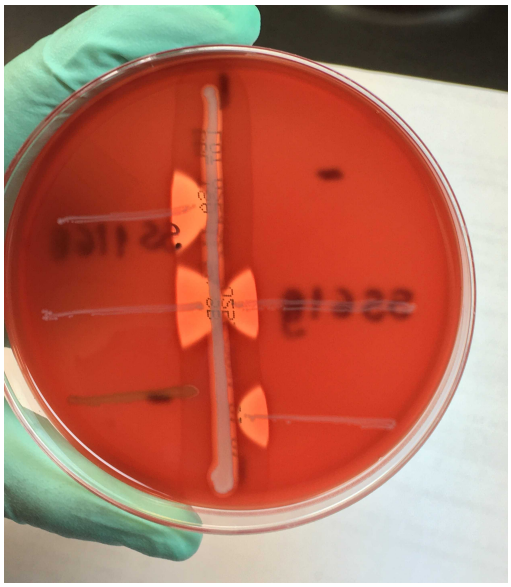

CAMP test.

Presence of triangular zone as a result of enhanced beta hemolysis was defined as CAMP test positive

Reference:
Carrol, KC, Pfaller MA, Landry, ML, Patel, R, McAdam, AJ, Richter, SS. In Streptococcus, 399-417. Manual of Clinical Microbiology. 12th ed. Washington DC: American Society for Microbiology Press; 2019.

To perform latex agglutination 30m

4

1. The latex agglutination is performed using PathDx Strep B latex – R62031.
2. Clean and label the slide. Prepare the bacterial suspension by placing a drop of 0.85% saline on the slide.
3. Harvest a heavy loop of fresh growth from BAP and mix with the saline on the slide in circular movements enough to make a milky suspension.
4. Homogenize/shake the PathDx Strep B flask, place one drop of the latex on top of the milky bacteria suspension and rock the slide for 1 minute. If agglutination occurs, the culture is positive.

Reference: According to the manufacturer's instructions

Storage of GBS isolates 1d

1d

5

After confirmed identification all GBS isolates should be frozen from a fresh culture (24 hours) in STGG or sheep blood and kept at -70°C for further testing (serotyping, antimicrobial susceptibility, etc…).

DNA fast extraction of *Streptococcus* 7h

7h

6

1. In a BSC, prepare the initial bacteria suspension. Depending on the organism being extracted, use the following amounts:
   - For *Streptococcus agalactiae*, using a 1µl loop, transfer 2-3 colonies of overnight blood agar plate growth to a labeled microcentrifuge tube containing 300µl of 0.85% NaCl and vortex.
2. Heat the suspension at 70°C for 15 minutes, use cap holder to secure the lid.
3. Spin microcentrifuge tube for 2 minutes in centrifuge at 10,000 rpm and remove supernatant.
4. Re-suspend the pellet in 50µl TE buffer, add 12µl mutanolysin (solution 2500U/ml) and 8µl of hyaluronidase (solution

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30mg/ml) and vortex.

- The mutanolysin solution is prepared by dissolving 10,000 units of mutanolysin (Sigma #M9901) into 4ml of PCR water (2500unit/ml stock solution); stored in aliquots of 500µl at -20°C.
- The hyaluronidase solution is prepared by dissolving 100mg hyaluronidase (Sigma #H3506) into 3.3ml PCR water (30mg/ml stock solution); stored in 500µl aliquots at -20°C.

5. Heat the suspension in water bath at 37°C for 30 minutes to overnight.
6. Heat the suspension to inactivate for 10 minutes in 100°C heat block, use cap holder to secure the lid.
7. Spin the tube for 4 minutes in centrifuge, identify the tube with the cryogenic label and use the DNA template in the supernatant.
8. Proceed immediately with PCR reaction or store lysates at -20°C until use.

da Gloria Carvalho M, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, O'Brien KL, Whitney CG, Cohen AL, Beall BW (2010). Revisiting Pneumococcal Carriage by Use of Broth Enrichment and PCR Techniques for Enhanced Detection of Carriage and Serotypes. Journal of Clinical Microbiology.
http://10.1128/JCM.02243-09

GBS serotyping by Conventional multiplex PCR 4h

7 4h

A. Mastermix preparation and PCR set up

1. Pipet 4.95µl PCR grade water, 12.5µl 2X Buffer-Qiagen Multiplex PCR Kit (catalogue number 206143) and 1uM of each primer per reaction in the labeled microcentrifuge PCR master mix tube (multiply the volumes by the number of reactions).
2. Vortex and spin briefly the tube with the prepared PCR master mix.
3. Pipet 22.5µl PCR master mix in each labelled 96-Well Plate/strip well. Add 2.5µl PCR grade water to the well labeled as negative control. Cover the wells containing the PCR master mix with 8-cap strips and take the 96-Well Plate to another biosafety cabinet, if possible, in another room.
4. Add 2.5µl of each DNA sample and S. agalactiae serotype DNA controls to the respective wells. Close the filled wells and press the caps tightly.
5. Enter the amplification parameters on the thermal cycler as follows:

95°C for 15 minutes	1 cycle
94°C for 30 seconds	35 cycles
54°C for 90 seconds	
72°C for 60 seconds	
72°C for 10 minutes	1 cycle
4°C until the machine is turned off	

6. Check the sample volume and review the parameters on the thermo cycler screen and start the run.

8 15m

2. Agarose gel preparation

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1. On a precision scale, weigh 1.6g Nusieve® agarose (catalogue number 50090).
2. Mix the weighed agarose with 80ml room temperature 1X TAE buffer (2% gel final concentration).
3. Heat in microwave for 90 seconds. Using thermoresistant gloves, carefully mix the solution, checking against the light to see if the agarose is completely dissolved, adding more time in the microwave as needed.
4. Let the agarose solution cool down to 50°C (about 5 minutes), add 5µl (0.5µg/µl) of EtBr.
5. Pour the gel on a casting tray with a 24 wells comb and wait until it solidifies. If not using the gel in the same day, cover the gel with 1X TAE buffer and put it in the refrigerator until ready to use.

9 2h

3. Gel loading and running

1. Carefully remove the comb and place the gel in the electrophoresis unity. Fill the unity with refrigerated 1X TAE buffer until it covers the gel completely.
2. Load 15µl ready to use 50-bp ladder (catalogue number 70538-3) on the first and last gel wells, following the template.
3. Mix 10µl amplified DNA with 1µl gel loading dye and load each, following the prearranged order on the template, in the gel wells.
4. Set the voltage source to 100V for 90 minutes and turn electrophoresis unity on.

10 15m

4. Gel picture and analysis

1. Gel picture:

1. When the run is finished, turn the electrophoresis unity off, remove the gel and take it to a UV source to take a picture.
2. The results on the gel picture should be compared to the *S. agalactiae* controls bands and the serotypes determined for the samples' band sizes, that must exactly match the controls' band sizes before assigning a serotype.
   Note:
   Non-specific bands have been occasionally detected when performing multiplex PCR testing on clinical specimens.

3. Gel Picture Example:

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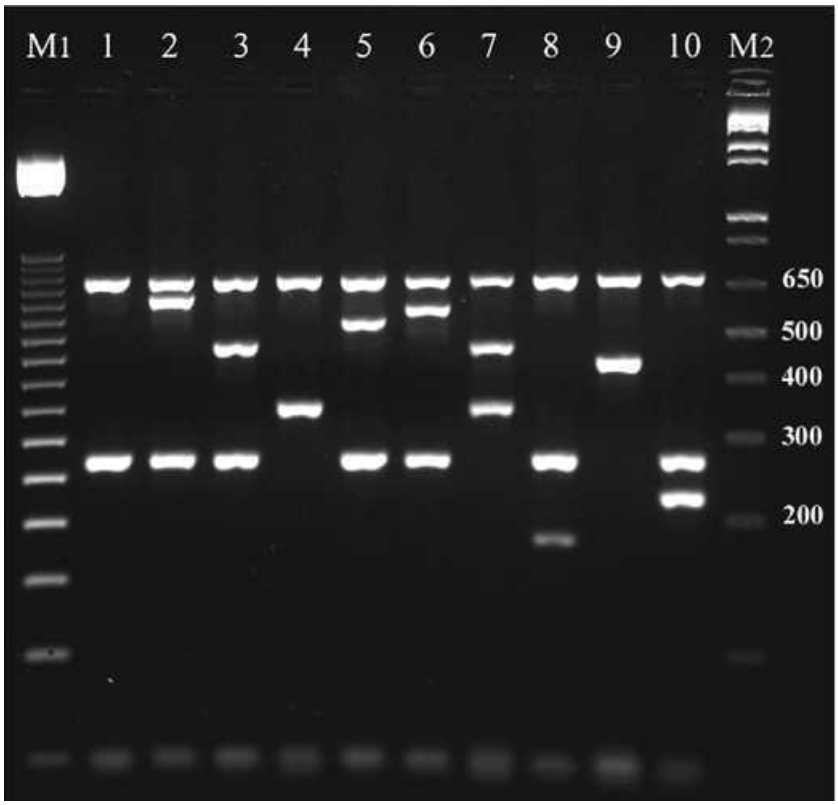

Gel analysis of the cmPCR amplification products:

M1: 50 bp Step Ladder; Lane 1*: S.agalactiae* serotype Ia; Lane 2: *S.agalactiae* serotype Ib;
Lane 3: *S.agalactiae* serotype II; Lane 4: *S.agalactiae* serotype III; Lane 5: *S.agalactiae* serotype IV;
Lane 6: *S.agalactiae* serotype V; Lane 7: *S.agalactiae* serotype VI; Lane 8: *S.agalactiae* serotype VII;
Lane 9: *S.agalactiae* serotype VIII; Lane 10: *S.agalactiae* serotype IX; M2: 1 kb plus DNA Ladder.

(Imperi et al, 2010, DOI: 10.1016/j.mimet.2009.11.010)

30m

11

5. Interpretation of Results

Serotype results are defined by the presence of specific bands and band sizes shown by gel electrophoresis of each sample.

1. Each sample must contain the 688 bp band (capsular gene cpsi) common to all serotypes.
2. Serotype specific band sizes are shown in the table below to determine serotype based on band sizes seen for sample.

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S. agalactiae serotype	Number of bands	Band sizes (bp)
Ia	2	272, 688
Ib	3	272, 621, 688
II	3	272, 465, 688
III	2	352, 688
IV	3	272, 538, 688
V	3	272, 582, 688
VI	3	352, 470, 688
VII	3	179, 272, 688
VIII	2	438, 688
IX	3	229, 272, 688

Imperi M, Pataracchia M, Alfarone G, Baldassarri L, Orefici G, Creti R (2010). A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of Streptococcus agalactiae.. Journal of microbiological methods.
https://doi.org/10.1016/j.mimet.2009.11.010

To perform antimicrobial susceptibility testing 2d

2d

12

Obtain BAP with overnight growth of isolate to be tested. Examine culture for purity. It should contain unique colony type with the expected *S. agalactiae* morphology. The test should be performed only with pure culture.

1. Using a sterile swab or a loop, transfer few colonies to 5 ml of saline or Mueller-Hinton broth. Vortex the suspension.
2. Use the 0.5 McFarland standard and/or densitometer to determine turbidity of suspension. Add cells to obtain the 0.5 McFarland standard (1.5×10^8 CFU/ml) suspension. The suspension should be used within 15 minutes.
3. Transfer 100 µl of the adjusted suspension to Mueller-Hinton broth with lysed horse blood. Mix the suspension gently by inversion and dispense into a sterile reservoir.
4. Open the S. agalactiae MIC panel package with a disinfected scissor; label the panel with isolate ID and transfer 100 µl of the inoculated Mueller-Hinton broth with lysed horse blood broth to each well using a multichannel pipette. After finish the whole panel inoculation cover the top of the panel with a plate seal to avoid evaporation. Note: Make sure every well is covered or evaporation can occur.
5. Incubate the inoculated MIC panel in ambient air at 35-37^{O}C for 20-24 hours. When the incubation period is complete read the MIC panel with the help of parabolic magnifying mirror and light. Record the antimicrobial endpoint on the work sheet as the first well showing no readable visible growth. The endpoint is the first well showing no readable visible growth (100% inhibition) for that antimicrobial. The MIC results should be interpretive according to latest CLSI recommendations.

Reference:
Clinical and Laboratory Standards Institute. CLSI Document M100-S22. edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

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