THE UNIVERSITY OF SYDNEY

NeoCol: Standard Operating Procedure (SOP) for GBS Serotyping & Antibiotic Susceptibility Testing V1.0

Purpose:

The purpose of this SOP is to describe the standard procedures involved in serotyping and antibiotic susceptibility testing for confirmed GBS isolates as part of NeoCOL study.

Principal:

The vagino-rectal swabs taken are first screened using chromogenic media to determine if positive or negative. Positive isolates are stored in STGG at -80^{o}C until ready for further analysis. This protocol provides a guideline for identification and antimicrobial susceptibility testing by microdilution of Group B Streptococcus (GBS) / Streptococcus agalactiae. Isolate DNA is obtained using the fast extraction methodology for *Streptococcus* isolates and serotyping of GBS isolates is to be performed by conventional multiplex PCR. Antimicrobial susceptibility testing by broth microdilution is to be performed following the recommendations of Clinical and Laboratory Standards Institute minimum inhibitory concentration (MIC) breakpoints.

Responsibility:

This SOP applies to any laboratory staff who are processing swabs for the NeoCOL study. It is the responsibility of those users to always follow these guidelines when processing swabs for the study.

Safety Requirements:

Gloves and a laboratory gown should be always worn during sample processing. Standard hand hygiene practices should be followed before, and after handling of samples. Handle all specimens with care and treat them as potentially infectious material.

Materials:

- Stored sample for testing (positive isolate stored in STGG at -80^{o}C)
- Sterile inoculation loops
- Blood agar
- Pipette and pipette tips
- Microcentrifuge tubes
- Centrifuge
- TE buffer
- Hyaluronidase
- PCR water
- Heat block
- Buffer-Qiagen Multiplex PCR Kit
- Thermo cycler
- Precision scale
- Nusieve® agarose
- Microwave
- Thermoresistant gloves
- Casting tray with a 24 well comb
- Electrophorese unit
- Mueller-Hinton broth
- Densitometer
- Lysed horse blood
- Multichannel pipette
- *S. agalactiae* MIC panel kit
- Parabolic magnifying mirror and light

Procedure:

DNA fast extraction of *Streptococcus*:

a. In a BSC, prepare the initial bacteria suspension. Depending on the organism being extracted, use the following amounts:
b. For *Streptococcus agalactiae*, using a 1µl loop, transfer 2-3 colonies of overnight blood agar plate growth to a labelled microcentrifuge tube containing 300µl of 0.85% NaCl and vortex.
c. Heat the suspension at 70^{o}C for 15 minutes, use cap holder to secure the lid.
d. Spin microcentrifuge tube for 2 minutes in centrifuge at 10,000 rpm and remove supernatant.
e. Re-suspend the pellet in 50µl TE buffer, add 12µl mutanolysin (solution 2500U/ml) and 8µl of hyaluronidase (solution 30mg/ml) and vortex.
    i. 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^{o}C.
    ii. 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^{o}C.
f. Heat the suspension in water bath at 37^{o}C for 30 minutes to overnight.
g. Heat the suspension to inactivate for 10 minutes in 100^{o}C heat block (use cap holder to secure the lid).
h. Spin the tube for 4 minutes in centrifuge, identify the tube with the cryogenic label and use the DNA template in the supernatant.
i. Proceed immediately with PCR reaction or store lysates at -20^{o}C until use.

GBS serotyping by Conventional multiplex PCR:

a. Mastermix preparation and PCR set up:
    i. Pipette 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 labelled microcentrifuge PCR master mix tube (multiply the volumes by the number of reactions).
    ii. Vortex and spin briefly the tube with the prepared PCR master mix.
    iii. Pipette 22.5µl PCR master mix in each labelled 96-Well Plate/strip well.
    iv. Add 2.5µl PCR grade water to the well labelled as negative control.
    v. 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.
    vi. 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.
    vii. Enter the amplification parameters on the thermo 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	

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

b. Agarose gel preparation:
   i. On a precision scale, weigh 1.6g Nusieve® agarose (catalogue number 50090).
   ii. Mix the weighed agarose with 80ml room temperature 1X TAE buffer (2% gel final concentration).
   iii. Heat in microwave for 90 seconds.
   iv. 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.
   v. Let the agarose solution cool down to 50°C (about 5 minutes), add 5µl (0.5µg/µl) of EtBr.
   vi. 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.

c. Gel loading and running:
   i. 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.
   ii. Load 15µl ready to use 50-bp ladder (catalogue number 70538-3) on the first and last gel wells, following the template.
   iii. Mix 10µl amplified DNA with 1µl gel loading dye and load each, following the prearranged order on the template, in the gel wells.
   iv. Set the voltage source to 100V for 90 minutes and turn electrophoresis unity on.

d. Gel picture and analysis
   i. When the run is finished: turn the electrophoresis unit off, remove the gel and take it to a UV source to take a picture.
   ii. The results on the gel picture should be compared to the *S. agalactiae* controls bands and the serotypes determined for the samples' band sizes must exactly match the controls' band sizes before assigning a serotype.

iii. Note: Non-specific bands have been occasionally detected when performing multiplex PCR testing on clinical specimens.

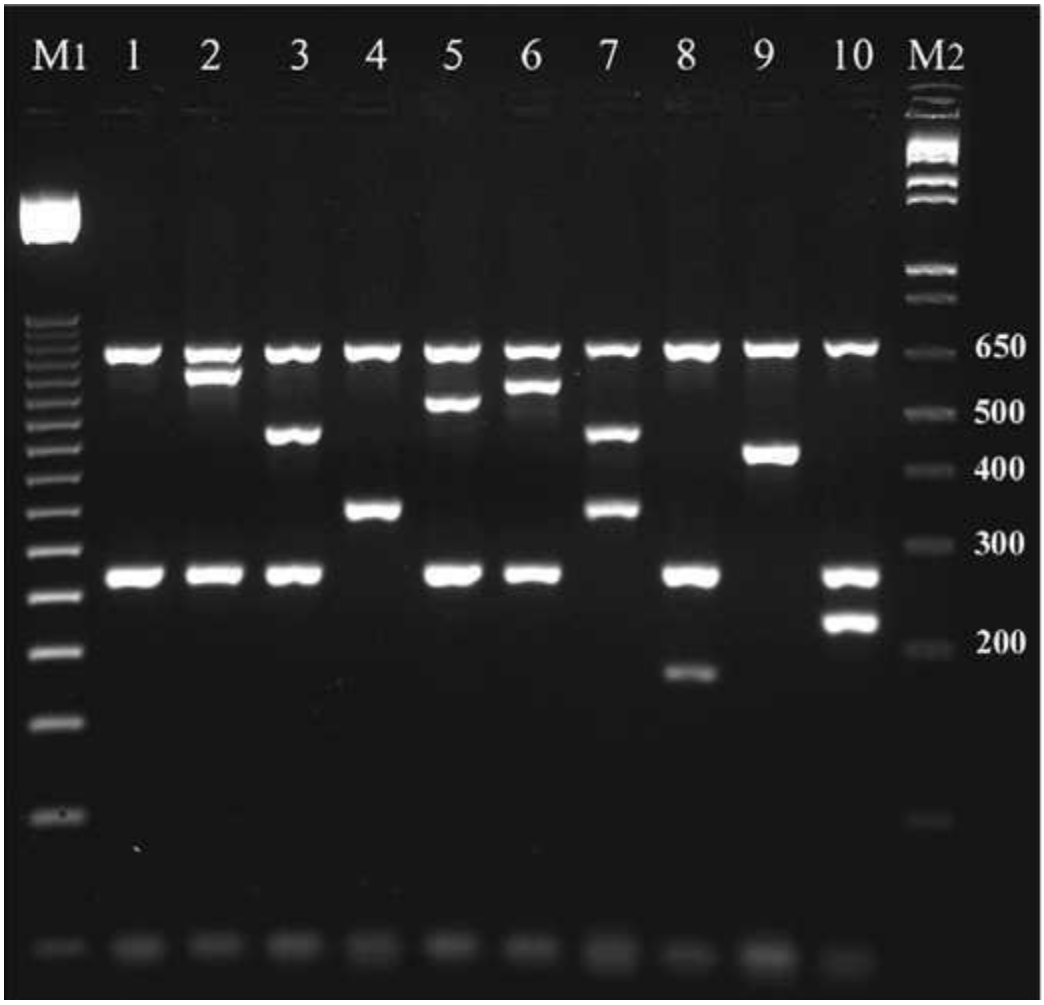

Figure 1: Example of gel

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.

e. Interpretation of Results

i. Serotype results are defined by the presence of specific bands and band sizes shown by gel electrophoresis of each sample.
ii. Each sample must contain the 688 bp band (capsular gene cpsi) common to all serotypes.
iii. Serotype specific band sizes are shown in the table below to determine serotype based on band sizes seen for sample.

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

To perform antimicrobial susceptibility testing:

a. 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.
b. Using a sterile swab or a loop, transfer few colonies to 5 ml of saline or Mueller-Hinton broth.
c. Vortex the suspension.
d. 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.
e. 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.
f. 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 finishing 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.
g. 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.

References

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

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

c. Group B Streptococcus (Streptococcus agalactiae) Isolation, Identification and Antimicrobial Susceptibility Testing. Online.

Document History

Version	Author(s)	Approved by	Update Reason	Date	SOP No:
1.0	Wisnu Tafroji, adapted by R. Kelleghan	P. Williams	Adapted for NeoCOL 2.0	26FEB2025	NeoCOL_2.0_SOP10

Site Training Record

Trainee Name	Read/Understand SOP (Tick)	Access to SOP (Tick)	Trainee Signature	Date	Trainer Initials