18/MHS06/039

MedLabSci

MCB202Assignment

1•

Streak-stab technique

OftenwheninoculatingaBAPtoobservehemoloysispatterns, investigatorswillalsostab

severaltimesthroughtheagarusinganinoculatingloop.Thisstaballowsforthedetectionof

streptolysinO,aspecifichemolysinproducedbyStreptococcuspyogenes.Thishemolysinis

inactivatedbyO2andisonlyseensubsurface(inananaerobicenvironment)aroundthestab

Mark.Notetheoval-shapedareasofclearingaroundthestabmarksinthepicturebelow;

thesearecausedbystreptolysinO.

MannitolSaltAgar(MSA)

Thistypeofmediumisbothselectiveanddifferential.TheMSAwillselectfororganismssuch

asStaphylococcusspecieswhichcanliveinareasofhighsaltconcentration(plateontheleft

inthepicturebelow).ThisisincontrasttoStreptococcusspecies,whosegrowthisselected

againstbythishighsaltagar(plateontherightinthepicturebelow).

ThedifferentialingredientinMSAisthesugarmannitol.Organismscapableofusingmannitol

asafoodsourcewillproduceacidicbyproductsoffermentationthatwilllowerthepHofthe

media.TheacidityofthemediawillcausethepHindicator,phenolred,toturnyellow.

Staphylococcusaureusiscapableoffermentingmannitol(leftsideofleftplate)while

Staphylococcusepidermidisisnot(rightsideofleftplate).

OxidaseTest

Thistestisusedtoidentifymicroorganismscontainingtheenzymecytochromeoxidase

(importantintheelectrontransportchain).Itiscommonlyusedtodistinguishbetween

oxidasenegativeEnterobacteriaceaeandoxidasepositivePseudomadaceae.

Cytochromeoxidasetransferselectronsfromtheelectrontransportchaintooxygen(thefinal

electronacceptor)andreducesittowater.Intheoxidasetest,artificialelectrondonorsand

acceptorsareprovided.Whentheelectrondonorisoxidizedbycytochromeoxidaseitturnsa

darkpurple.Thisisconsideredapositiveresult.Inthepicturebelowtheorganismontheright

(Pseudomonasaeruginosa)isoxidasepositive.

Coagulasetest

Coagulaseisanenzymethatclotsbloodplasma.ThistestisperformedonGram-positive,

catalasepositivespeciestoidentifythecoagulasepositiveStaphylococcusaureus.Coagulase

isavirulencefactorofS.aureus.Theformationofclotaroundaninfectioncausedbythis

bacterialikelyprotectsitfromphagocytosis.ThistestdifferentiatesStaphylococcusaureus

fromothercoagulasenegativeStaphylococcusspecies.

TaxosA(bacitracinsensitivitytesting)

Thisisadifferentialtestusedtodistinguishbetweenorganismssensitivetotheantibiotic

bacitracinandthosenot.BacitracinisapeptideantibioticproducedbyBacillussubtilis.It

inhibitscellwallsynthesisanddisruptsthecellmembrane.Thistestiscommonlyusedto

distinguishbetweentheb-hemolyticstreptococci:Streptococcusagalactiae(bacitracin

resistant)andStreptococcuspyogenes(bacitracinsensitive).Theplatebelowwasstreaked

withStreptococcuspyogenes;noticethelargezoneofinhibitionsurroundingthedisk.

TaxosP(optochinsensitivitytesting)

Thisisadifferentialtestusedtodistinguishbetweenorganismssensitivetotheantibiotic

optochinandthosenot.ThistestisusedtodistinguishStreptococcuspneumoniae(optochin

sensitive(picturedontherightbelow))fromothera-hemolyticstreptococci(optochin

resistant(Streptococcusmitisispicturedontheleftbelow)).

Ureasetest

Thistestisusedtoidentifybacteriacapableofhydrolyzingureausingtheenzymeurease.Itis

commonlyusedtodistinguishthegenusProteusfromotherentericbacteria.Thehydrolysisof

ureaformstheweakbase,ammonia,asoneofitsproducts.ThisweakbaseraisesthepHof

themediaabove8.4andthepHindicator,phenolred,turnsfromyellowtopink.Proteus

mirabilisisarapidhydrolyzerofurea(centertubepicturedhere).Thetubeonthefarrightwas

inoculatedwithaureasenegativeorganismandthetubeonthefarleftwasuninoculated.

Motilityagar

isadifferentialmediumusedtodeterminewhetheranorganismisequippedwithflagellaand

thuscapableofswimmingawayfromastabmark.Theresultsofmotilityagarareoften

difficulttointerpret.Generally,iftheentiretubeisturbid,thisindicatesthatthebacteriahave

movedawayfromthestabmark(aremotile).Theorganismsinthetwotubespicturedonthe

rightaremotile.If,however,thestabmarkisclearlyvisibleandtherestofthetubeisnot

turbid,theorganismislikelynonmotile(tubepicturedontheleft).

SpiritBlueagar

Thisagarisusedtoidentifyorganismsthatarecapableofproducingtheenzymelipase.This

enzymeissecretedandhydrolyzestriglyceridestoglycerolandthreelongchainfattyacids.

Thesecompoundsaresmallenoughtopassthroughthebacterialcellwall.Glycerolcanbe

convertedintoaglycolysisintermediate.Thefattyacidscanbecatabolizedandtheir

fragmentscaneventuallyentertheKreb’scycle.Spiritblueagarcontainsanemulsionofolive

oilandspiritbluedye.Bacteriathatproducelipasewillhydrolyzetheoliveoilandproducea

haloaroundthebacterialgrowth.TheGram-positiverod,Bacillussubtilisislipasepositive

(picturedontheright)Theplatepicturedontheleftislipasenegative.

Simmon’sCitrateAgar

Thisisadefinedmediumusedtodetermineifanorganismcanusecitrateasitssolecarbon

source.ItisoftenusedtodifferentiatebetweenmembersofEnterobacteriaceae.Inorganisms

capableofutilizingcitrateasacarbonsource,theenzymecitrasehydrolyzescitrateinto

oxaoloaceticacidandaceticacid.Theoxaloaceticacidisthenhydrolyzedintopyruvicacidand

CO2.IfCO2isproduced,itreactswithcomponentsofthemediumtoproduceanalkaline

compound(e.g.Na2CO3).ThealkalinepHturnsthepHindicator(bromthymolblue)from

greentoblue.Thisisapositiveresult(thetubeontherightiscitratepositive).Klebsiella

pneumoniaeandProteusmirabilisareexamplesofcitratepositiveorganisms.Escherichiacoli

andShigelladysenteriaearecitratenegative.

2•

Stainingtechniques

GramStaining

Gramstainisakeystartingpointtoidentifymicrobialspecies.Thestaindifferentiatesmembranestructuresbetweengram-

positiveandgram-negativemicroorganisms.Gram-positive

microbeshaveathickcellwallmadeupofpep-tidoglycan(50–90%),whicharestainedpurple

bycrystalviolet,whereasgram-negativemicrobeshaveathinnerlayer(10% ofcellwall),

whicharestainedpinkbythecounter-stainsafranin[24,30,33–36].

1.Applytwodropsofcrystalvioletonsmearfor30s.

2.Washwithtapwater.

3.AddtwodropsofGram’siodinefor30s.

4.Repeatstep2.

5.Add95% ethanol.

6.Repeatstep2.

7.Addtwodropsofsafranin.

8.Repeatstep2.

9.Observeinmicroscopeunderoilimmersion.10.Yeastsaregram-positive,butpoorlystained;

Cryptococcusneoformansisanotableexception(

gram-negative).

GiemsaStaining

Avarietyof“Romanowsky-type”stainswithmixturesofmethyleneblueandazureeosincompoundshavebeenusedsuccessfullyformanyyearsondiversefungiwithvariousprocedures

andmodifications.GiemsastainisamemberoftheRomanowskygroupofstains,whichare

definedasbeingtheblackprecipitateformedfromtheadditionofmethanol[37].Inthisstain,

eosinionsarenegativelychargedandstainbasiccomponentsofcellsorangetopink.Itwas

alsooriginallydesignedtoincorporatecytoplasmic(pink)stainingwithnuclear(blue)staining

andfixationasasinglestepforsmearsandthinfilms.Thisstainhaswidelybeenusedto

examinePneumocystisjiroveci,Rhinosporidiumseeberi,andHistoplasmacapsulatum[38–40].

1.Floodthesmearwithmethylalcoholandleavefor3–5minforfixation.

2.AddpreparedGiemsastainandleavefor45min.

3.Washslidethoroughlywithrunningtapwater.

4.Blotdrywithabsorbentpaper.

5.Observeunderoilimmersion.

6.Lookforintracellularbuddingyeasts;fungi

stainwithpurplish-blue.

WrightStaining

TheWrightstainisanalcoholicsolutionofmeth-yleneblue,azureA,thionin,andeosinY.

Methylgroupsareactivatedandreactwithchargedcom-ponentsofthecelltoproduce

coloration.Itisusedtodetectbloodparasites,viralandchlamyd-ialinclusionbodies,yeast

cells,andspeciesofPneumocystis.Eosinionsarenegativelychargedandstainbasic

componentsofcellsorangetopink,whereasotherdyesstainacidiccellstruc-turestovarious

shadesofbluetopurple1.CoverthesmearwithfreshlyfilteredWright

stainandleavefor1–3min.

2.Withoutremovingthestain,pouronbuffer

solution(pH)Gentlymixbufferandstain;uponpropermix-ing,metallicgreensheen(green

scum)risestothesurfaceofthefluid.

4.Leavefor3minorlonger.

5.Washtheslidegentlywithflowingtapwater

andwipethebottomoftheslidewithaclean

filterpaper.

6.Air-drytheslideandobserveunderthe

microscope.

7.Intracellularyeastcellsaretypicallystainblue

andspeciesofPneumocystisstainpurple.

2b

Identificationtechniques

WetMountTechniques

Fungalspecimenscanbevisualizedusingwetmounttechniquesthroughsuspensionof

cultureineitherwaterorsaline,mixedwithalkalitodis-solvebackgroundmaterial[21]or

mixedwithacombinationofalkaliandcontrastingdye(e.g.,lactophenolcottonblueorIndia

ink)[22,23].

19StainingTechniquesandBiochemicalMethodsfortheIdentificationofFungi241

Thedyesnonspecificallystainthefungalmate-rial,whichincreasescontrastwiththe

backgroundandpermitsexaminationofthedetailedstruc-tures.AvariationistheIndiaink

method,inwhichtheinkdarkensthebackgroundratherthanthefungi.

PotassiumHydroxideWetMount

Potassiumhydroxide(KOH)isusedtodissolveproteinaceousmaterialandfacilitatedetection

offungalelementsthatarenotaffectedbystrongalkalisolution.Itisastrongalkaliusedasa

clear-ingagenttoobservefungiinawetmountprepa-ration.TheconcentrationofKOHis

usuallybasedonthespecimenthatisbeingused.Normally,10–20% KOHisused;occasionally,

40% isusedwhenthespecimenisnotclearedby10–20% ofKOH.Inthismethod,thefungal

structures,suchashyphae,largeyeasts(Blastomyces),spherules,andsporangia,arewell

distinguished.Inunstainedpreparations(KOHwithoutstain),thefungalstructuresmaybe

enhancedbyusingaphasecon-trastmicroscope[18,24].Theclearingeffectthroughoutthe

specimencanbeacceleratedbygentlyheatingtheKOHpreparation.

Visualizationoffungicanbefurtherenhancedbytheadditionofdyestothepreparation.This

methodisquick,simple,andinexpensive[25,26].

1.PlacealargedropofKOHsolutionwitha

Pasteurpipette.

2.Transfersmallquantityoftheculturewitha

looporthetipofascalpelintotheKOHdrop.

3.Putacleancoverslipoverthedropgentlyso

thatnoairbubbleistrapped.

4.Clearingcanbehastenedbygentleheatingof

theslide,butitisbestavoided.

5.Observeunder20×and40×objectiveoflight

orphasecontrastmicroscope.

6.Lookforbuddingyeastcells;branching

hyphae;typeofbranching;andthecolor,sepa-ration,andthicknessofhyphae(seeNote12).

LactophenolCottonBlueWetMount

Lactophenolcottonblue(LCB)isamountingmediumcommonlyusedinmicrobiologylaboratoriesforpreparingmountsoffungalcultures.

LCBisusedasbothmountingfluidandstain.

Inthismethod,phenolwillkilltheorganisms,and

thelacticacidpreservesfungalstructures;chitininthefungalcellwallisstainedbythecotton

blue.ItcanbeusedaloneorinconjunctionwithKOH.Libraryslidesmaybemadebyallowing

themounttodryfor3weeksandthensealingwithcollodion[24,27,28].

1.PutalargedropofLCBwithaPasteurpipette.

2.Transferasmallquantityoftheculturetothedrop.

3.Teasetheculturewellwithteasingneedles,soastogetauniformspread.

4.Putonacoverslipgentlytoavoidentrapmentofairbubbles.

5.Examineunderthe20×and40×objectivesoflightmicroscope.

6.Observethemorphologicalfeaturescarefully.7.Fungalelementswillstaindeepblue

againsta

clearpale-bluebackground.

IndiaInkWetMount

Indiainkcanbeaddedtospecimenstoprovidedarkbackgroundthatwillhighlighthyaline

yeastcellsandcapsularmaterial.Thismethodisusedtodetectmicroorganismsthatare

surroundedbycapsules.Thedyeisexcludedbythecapsule,cre-atingaclearhaloaroundthe

yeastcell.Itisarapidmethodforthepreliminarydetectionandidentificationofspecimens

containingspeciesofCryptococcus[29].

1.AddasmalldropofIndiainkonasmear.

2.Placeacoverslipoverthesmearandpressit

gentlytoobtainathinmount.

3.IfIndiainkistoothick(dark),diluteitby50%

withsaline.

4.Allowthepreparationtostandforfewminutes

tosettle.

5.Scanunderlowpowerinreducedlight;switch

tohighpower,ifnecessary.

6.Organismspossessingacapsuleappearhighly

refractile,surroundedbyaclearzoneagainstadarkbackground