TECHNICAL MANUAL

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ALA (RAPID PORPHYRIN TEST)

Principle

This test is used for rapidly detecting porphyrin as a means of speciating *Haemophilus* species. Enzymes which convert ALA (delta - aminolevulinic acid) to porphyrins in the biosynthesis of hemin (X factor) are produced by *Haemophilus parainfluenzae* but not by *H. influenzae*. The production of porphyrins is detected by examination with an ultra-violet (UV) light.

Reagents

BBL TAXO Differentiation Disks ALA. (Store refrigerated in the dark. Allow 10-15 minutes for the container to reach room temperature before opening).
Sterile distilled water

Other Materials

Petri dish
Inoculating loop
Gauze
Long-wave UV lamp
Forceps

Procedure

1. Place one ALA disk for each organism to be tested on the inside of a Petri dish using forceps.
2. Moisten each disk with one drop of sterile water.
3. Rub a loopful of the test organism onto the moistened disk holding it in place with sterile forceps.
4. Saturate gauze with water, squeeze out any excess and place it in the petri dish as far away from the disks as possible.
5. Incubate at 35^{o}C.
6. Examine at hourly intervals for 6 hours by removing the top of the petri dish and exposing the disks to UV light in a darkened room. **NB:** Wear UV safety goggles when using the UV light.

Interpretation

A. Positive: Orange-red fluorescence

B. Negative: No fluorescence observed

Precautions

1. Use for differentiating *Haemophilus* spp. only.

2. Best results are obtained when a heavy inoculum is used.

3. ALA is light sensitive. Disks must be protected from light.

Quality Control

Test the following positive and negative controls each time an unknown is tested:

Positive: *H. parainfluenza* (ATCC 7901)
Negative: *H. influenzae* (ATCC 35056)

Reference

BBL TAXO Differentiation Disks ALA package insert, 1999. Becton Dickinson Microbiology Systems.

ANAEROBE IDENTIFICATION USING SPECIAL-POTENCY DISKS

Principle

Special potency antimicrobial disks of vancomycin (5 μg), kanamycin (1,000 μg) and colistin (10 μg) are used as an aid in determining the Gram reaction of anaerobes as well as in preliminary categorization of some anaerobic genera and species (Table 1). In general, gram positive organisms are resistant to colistin and susceptible to vancomycin, while most gram negative organisms are resistant to vancomycin. This difference is especially useful with some Clostridia that consistently stain gram negative.

Table 1 - Anaerobic Identification by Means of Special Potency Disks

	Response[1] to Disk:		
Type of Organism	**Vancomycin (5 μg)**	**Kanamycin (1,000 μg)**	**Colistin (10 μg)**
Gram negative	R	V	V
Gram positive	S	V	R
B. fragilis group	R	R	R
B. ureolyticus group	R	S	S
Fusobacterium spp.	R	S	S
Porphyromonas spp.	S	R	R
Veillonella spp.	R	S	S

[1] R - resistant; S - susceptible; V - variable

Materials

A. Reagents

1. Special potency antibiotic disks:

Vancomycin	5 μg
Kanamycin	1,000 μg
Colistin	10 μg

Store a small supply of disks (one carton each) in a tight container with desiccants at 4^0C.

2. Brucella or other anaerobic blood agar plate.

B. Supplies

1. Single disk dispenser or forceps
2. Ruler (divided into millimeters)

Procedure

1. Allow the container with disks to reach room temperature before opening it.
2. Subculture the isolate on a BAP. To ensure an even, heavy lawn of growth, streak the first quadrant back and forth several times. Streak the other quadrants to yield isolated colonies.
3. Place the three antibiotic disks on the first quadrant will apart from each other.
4. If you have several organisms to test, first streak all the plates and then add the disks to them at the same time.
5. Incubate the plate(s) anaerobically for 48-72 hours at 35-37^0C.
6. Examine for zones of inhibition of growth around the disks.

Interpretation

A. Susceptible: Zone of inhibition of ≥ 10 mm
B. Resistant: Zone of inhibition of < 10 mm

Quality Control

A. Test special potency antibiotic disks by lot when initially received and weekly thereafter.

B. Test *Bacteroides fragilis* (ATCC 25285), *Clostridium perfringens* (ATCC 13124), and *Fusobacterium necrophorum* (ATCC 25286) as described below under Procedure. The results should show the following:

1. *B. fragilis*: resistant to all three antibiotics
2. *F. necrophorum*: resistant to vancomycin; susceptible to colistin and kanamycin
3. *C. perfringens*: susceptible to vancomycin and kanamycin and resistant to colistin

C. Record the results on a QC log.

SPS Disk for Differentiation of Anaerobic Cocci

Principle

Sodium polyanethol sulfonate (SPS), a commonly used anticoagulant, inhibits certain bacteria such as *Peptostreptococcus anaerobius* and the aerobe *Gardnerella vaginalis*. Paper disks impregnated with 5% SPS can be used as a tool for differentiating *P. anaerobius* from other anaerobic cocci.

Materials

A. Reagents

1. SPS Disks

   a. Combine the following in a flask.

SPS	5 g
Distilled water	100 ml

   b. After dissolving SPS, sterilize the mixture by filtration (0.22 μm pore size filter).
   c. Dispense 20 μl onto sterile 1/4-inch diameter filter paper disks that are spread inside empty, sterile petri dishes. Allow these to dry for 72 hours at room temperature.
   d. Store the disks at room temperature, and label with an expiration date of 6 months.

2. SPS disks are also commercially available (Anaerobe Systems, Difco, Oxoid, Remel). Store as indicated by the manufacturers.
3. Brucella or other anaerobic blood agar plate.

B. Supplies

1. Single-disk dispenser or forceps
2. Ruler (divided into millimeters)

Procedure

1. Allow the container with disks to reach room temperature before use.
2. Subculture the isolates on a BAP. To ensure an even, heavy lawn of growth, streak the first quadrant back and forth several times. Streak the other quadrants to yield isolated colonies.

3. Place the SPS disk on the first quadrant.
4. If you have several organisms to test, first streak all the plates and then add the disks to them at the same time. You can use one plate for up to four tests.
5. Incubate the plate(s) anaerobically for 48-72 hours at 35-37^{0}C.
6. Examine for a zone of inhibition of growth around the disk.

Interpretation

A. Susceptible: Zone of inhibition of ≥ 12mm

P. anaerobius usually gives a very large zone of inhibition (≥ 16 mm), whereas other anaerobic cocci that appear susceptible to SPS give smaller zones. To presumptively identify *P. anaerobius*, you must also consider the Gram stain, typical colonial morphology, and odor. Some strains of *P. micros* may be susceptible to SPS. Examine the Gram stain for the small cell size of *P. micros* and chaining characteristic of *P. anaerobius.*

B. Resistant: Zone of inhibition of <12 mm.

Quality Control

A. Test each lot upon receipt and monthly thereafter.
B. Test *P. anaerobius* ATCC 27337 and *Peptostreptococcus asaccharolyticus* ATCC 29745 as described below under Procedure. The results should show the following:
   1. *P. anaerobius*: susceptible to SPS
   2. *P. asaccharolyticus*: resistant to SPS
C. Record the results on a QC log.

ANAEROBIC/CAMPYLOBACTER JAR SET UP

Anaerobic Jar

1. Anaerobic plates are kept in the nitrogen holding box until there is enough for a jar or until the end of the day.

2. Place the inoculated plates (max 14), biological indicators and anaerobic indicator strip into an empty anaerobic jar.

3. Tear open an AnaeroGen foil sachet at the tear-nick indicated and remove the Anaero Gen paper sachet from within.

4. Immediately place the paper sachet in the jar down the side of the plates.

5. Place the lid on the jar (no catalyst required) and seal with the clamp.
   Note: The time between opening the foil sachet and sealing the jar should not exceed one minute.
   Note: Jar and lid must be labelled with the same number.

6. Label jar with date and place in walk in incubator.

Control Testing

An anaerobic indicator is added to each jar as it is set up to visually check that anaerobic conditions have been achieved and maintained. Check the jar after 2 hours incubation to make sure the indicator does not indicate oxygen present.

Biological Indicator

Inoculate a quarter anaerobic plate with the following test organisms:

Bacteroides fragilis ATCC 25285: growth
Clostridium perfringens ATCC 13124: growth
Clostridium difficile ATCC 9089: growth
Pseudomonas aeruginosa ATCC 27853: no growth

Record results on the anaerobic jar QC sheet.

Campylobacter Jar

1. Campylobacter plates are kept in the CO_2 incubator until there is enough for a jar or until 4 p.m. (Any late cultures will be set up at the end of the shift).

2. Place a dampened paper towel into the bottom of an anaerobic jar.

3. Place the inoculated plates (max 14) into the jar. Include a plate freshly inoculated with the control organism.

4. Tear open an CampyGen foil sachet at the tear-nick indicated and remove the CampyGen paper sachet from within.

5. Immediately place the paper sachet in the jar down the side of the plates.

6. Place the lid on the jar (no catalyst required) and seal with the clamp.
   Note: The time between opening the foil sachet and sealing the jar should not exceed one minute.
   Note: Jar and lid must be labelled with the same number.

7. Label jar with date and place in walk in 42^{o}C incubator.

Biological Indicator

Inoculate a campylobacter agar plate with the following test organism:

Campylobacter jejuni ATCC 29428: growth

Record results on the anaerobic jar QC sheet.

Note: The technologist on the enteric bench is responsible for the daily subculturing of the control organism (3 new plates). One newly subcultured plate will be incubated with the reincubate culture jar. The old control plate and the remaining 2 newly subcultured plates will be kept in the CO_2 incubator until the end of the day.

The 2 new subcultured plates are for setting up new jars. If more are needed, the technicians will subculture new plates from the old control plate.

IDENTIFICATION OF *CORYNEBACTERIUM* (API CORYNE)

Principle

The API CORYNE system facilitates the 24 hour identification of *C. jeikeium* (CDC Group JK), other medically important Corynebacteria, *Rhodococcus equi*, *Listeria* species, *Erysipelothrix rhusiopathiae*, Actinomyces pyogenes, Arcanobacterium haemolyticum, *Brevibacterium* species and *Gardnerella vaginalis*.

The API CORYNE strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of carbohydrates (CHO). The addition of a dense test suspension of bacteria rehydrates the enzymatic substrates. The metabolic end products produced during incubation are detected through spontaneous coloured reactions or by the addition of reagents.

The fermentation tests are inoculated with an enrichment medium (containing pH indicator) which reconstitutes the CHO substrates. Fermentation of CHO is detected by colour change in the pH indicator.

Materials

API Coryne strips - store at 2 - 8^0C
GP medium - store at 2 - 8^0C
McFarland Standard #6
Nitrate A - store at Room Temperature
Nitrate B - store at 2 - 8^0C
Zym A - store at 2 - 8^0C in the dark
Zym B - store at 2 - 8^0C in the dark
PYZ - store at 2 - 8^0C in the dark
H_2O_2 - store at 2 - 8^0C
Mineral oil
Sterile saline 3 ml

Procedure

1. Preparation of Inoculum

   a) Only pure cultures of a single organism should be used (heavily inoculated sheep BAP x 3; incubate for 24 hours at 35^0C in 5% CO_2).

   b) Using a sterile swab, harvest all the culture from 3 BAP and inoculate into 3 ml. sterile saline to give a turbidity of at least McFarland #6.

2. Preparation of the Strip

   a) An incubation tray is supplied for each strip.

   b) Dispense 5 ml of water into the wells of the tray.

3. Inoculation of the Strip

   a) Inoculate tests 1 $\rightarrow$ 11 of the strip (NIT to GEL).

   b) Avoid bubbles by tilting the strip slightly forward while placing the pipette tip on the side of the cupule.

   c) Add 3 drops into each cupule for tests NIT to ES.

   d) For the URE test fill the tube portion only.

   e) For the GEL test, fill both the tube and cupule. Then:

   f) For the last nine tests of the strip (O to GLYG transfer the rest of the bacterial suspension to an ampoule of GP medium. Mix well.

   g) Distribute the new suspension into the tubes only of tests O to GLYG

   h) Overlay cupules URE and O to GLYG with mineral oil, forming a slight convex meniscus.

   i) Cover with incubation lid and incubate the strip for 24 hours at 35^0C (non-CO_2).

Interpretation

REACTIONS TABLE

TESTS	REACTONS	NEGATIVE RESULTS	POSITIVE RESULTS
NIT	NITrate reduction	**NIT A + NIT B**	(10 mn)
		Colourless Very pale pink	Dark pink Red
PYZ	PYraZinamidase	**PYZ**	(10 mn)
		Colourless Very pale brown Very pale orange	Brown Orange
PyrA	Pyrrolidonyl Arylamidase	**ZYM A**	**+ZYM B**
		PyrA → B	NAG (10 mn)
		Colourless Pale orange	Orange
PAL	Alkaline Phosphatase	Colourless Beige-pale purple Pale orange	Purple
βGUR	Beta GlucURonidase	Colourless Pale grey Pale beige	Blue
βGAL	Beta GALactosidase	Colourless Beige-pale purple	Purple
∝ GLU	Alpha GLUcosidase	Colourless Beige-pale purple Pale green	Purple
BNAG	N-Acetyl-B Glucosaminidase	Colourless Beige-pale purple Pale brown Pale grey	Brown

REACTIONS TABLE (Cont'd)

TESTS	REACTONS	NEGATIVE RESULTS	POSITIVE RESULTS
ESC	ESCulin (β Glucosidase)	Colourless Grey	Black
URE	UREase	Yellow Orange	Red Pink
[GEL]	GELatine (hydrolysis)	No diffusion of black pigment	Diffusion of black pigment
O	**Control (Fermentation)**		
GLU	GLUcose }		
RIB	RIBose }		
XYL	XYLOSE }		
MAN	MANnitol } Fermentation		
MAL	MALtose }	Red	Yellow
LAC	LACtose }		
SAC	Sucrose }	Orange	Yellow-orange
GLYG	GLYcoGen }		
CAT	CATalase (ESC or GEL test)	H_2O_2 3%	1 min
		No bubbles	Bubbles

References

1. Coyle, Marie B., Benjamin Lipsky. 1990. Coryneform Bacteria in Infectious Diseases: Clinical and Laboratory Aspects. Clinical Microbiology Reviews. 3:227-246.

2. Freney, J.M.T. Duperron, C. Couturier, W. Hansen, F. Allard, J.M. Boueufgras, D. Monget, J. Fleurette. Evaluation of API Coryne in Comparison with Conventional Methods for Identifying Coryneform Bacteria, Journal of Clinical Microbiology, January 1991, Vol. 29, p. 38-41.

3. Murray P.A., et al. Manual of Clinical Microbiology, 7th ed., 1999.

IDENTIFICATION OF *ENTEROBACTERIACEAE* (API 20E)

Principle

The API 20E system facilitates the 24-hour identification of *Enterobacteriaceae* as well as 24 or 48-hour identification of other Gram negative bacteria.

The API 20E strip consists of microtubes containing dehydrated substrates for the demonstration of enzymatic activity and carbohydrate (CHO) fermentation. The substrates are reconstituted by adding a bacterial suspension. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. CHO fermentation is detected by colour change in the pH indicator.

Materials

API 20E strips - store at 2-8^{0}C
0.85% sterile saline
Nitrate A - store at 2-8^{0}C
Nitrate B - store at 2-8^{0}C
Mineral oil
Zinc dust
Kovacs Reagent
Voges - Proskauer Reagents
Ferric Chloride
H_2O_2
Oxidase Reagent

} Store at 2-8^{0}C

OF Dextrose
Motility Medium

} ID of non-*Enterobacteriaceae*

Procedure

1. Preparation of Inoculum

   a) Add 5 ml. of 0.85% saline to a sterile test tube.

   b) Using a sterile inoculating loop, carefully touch the centre of a well isolated colony (2-3 mm. Diameter) and thoroughly emulsify in the saline.

2. Preparation of the Strip

   a) An incubation tray and lid is supplied for each strip.
   b) Dispense 5 ml of water in to the tray.

3. Inoculation of the Strip

   a) Remove the cap from the tube containing the bacterial suspension and insert a 5 ml. Pasteur pipette.
   b) Tilt the API 20E incubation tray and fill the tube section of the microtubes by placing the pipette tip against the side of the cupule.

      Note: The ADH, LDC, ODC, H2S, AND URE reactions can be interpreted best if these microtubes are slightly underfilled.
   c) Fill both the TUBE and CUPULE section of [CIT], [VP] and [GEL] tubes.
   d) After inoculation, completely fill the cupule section of the ADH, LDC, ODC, H2S and URE tubes with mineral oil.
   e) Using the excess bacterial suspension, inoculate an agar slant or plate (non-selective media such as nutrient agar, blood agar or tryptic (trypticase) soy agar is suggested) as a purity check and for oxidase testing, serology, and/or additional biochemical testing. Incubate the slant or plate for 18-24 hours at 35^0C.

4. Incubation of the Strip

   a) After inoculation, place the plastic lid on the tray and incubate the strip for 18-24 hours at 35^0C in a non-CO_2 incubator.

   b) Weekend incubation: The biochemical reactions of the API 20E should be read after 18-24 hours incubation. If the strips cannot be read after 24 hours incubation at 35^0C, the strips should be removed from the incubator and stored at 2-8^0C (refrigerator) until the reactions can be read.

5. Reading the Strip

   a) After 18 hours of incubation and before 24 hours incubation, record all reactions not requiring the addition of reagents.

   b) If the GLU tube is negative (blue or green), do not add reagents. Reincubate a further 18-24 hours.

c) If the GLU is positive (yellow):

i. Perform the oxidase test.

A portion of the growth from the agar slate or plate, inoculated from the 20E bacterial suspension, should be rubbed onto filter paper to which a drop of oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) has been added. The area where the growth has been added will turn dark purple within 10 seconds if the reaction is positive and will be colourless or light purple if negative.

Note: (a) Nichrome wire loops should NOT be used in performing the oxidase test. Nichrome wire can cause a false positive reaction.

(b) The oxidase test should NOT be performed using bacterial growth from selective media such as MacConkey, EMB, etc.

Note: (a) Before addition of reagents, observe GLU tube (positive or negative) for bubbles.

(b) The nitrate reduction and indole tests must be performed last since these reactions release gaseous products which interfere with the interpretation of other tests on the strip. The plastic incubation lid should not be replaced after the addition of these reagents.

ii. Add the reagents to TDA and VP tubes. If positive, the TDA reactions will be immediate, whereas the VP reaction may be delayed up to 10 minutes.

iii. The Kovacs' reagent should then be added to the IND tube.

iv. The Nitrate Reduction test should be performed on all oxidase positive organisms. The reagents should be added to the GLU tube after the Kovacs Reagent has been added to the IND tube.

Interpretation

a) Use the API 20E analytical profile index. (For 18-24 hour tests, use white pages. For 36-48 hour tests, use blue pages.)

b) The tests are separated into groups of three. The following numerical value is assigned to each reaction recorded:

1- positive reaction in the first test of the group
2- positive reaction in the second test of the group
4- positive reaction in any test
0- negative reaction in any test

Reference

1. Murray P.A., et al. Manual of Clinical Microbiology, 7th ed., 1999.

SUMMARY OF RESULTS - 18-24 HOUR PROCEDURE

TUBE	INCUBATION	POSITIVE	NEGATIVE	COMMENTS
ONPG		Yellow	Colourless	(1) Any shade of yellow is a positive reaction. (2) VP tube, before the addition of reagents, can be used a negative control.
ADH	18-24 hr 36-48 hr	Red or Orange Red	Yellow Yellow or Orange	Orange reactions occurring at 36-48 hours should be interpreted as negative.
LDC	18-24 hr 36-48 hr	Red or Orange Red	Yellow Yellow or Orange	Any shade of orange within 18-24 hours is a positive reaction. At 36-48 hours, orange decarboxylase reactions should be interpreted as negative.
ODC	18-34 hr 36-48 hr	Red or Orange Red	Yellow Yellow or Orange	Orange reactions occurring at 36-48 hours should be interpreted as negative.
CIT		Turquoise or Dark Blue	Light Green Or Yellow	(1) Both the tube and cupule should be filled. (2) Reaction is read in the aerobic (cupule) area.
H_2S		Black Deposit	No Black Deposit	(1) H_2S production may range from a heavy black deposit to a very thin black line around the tube bottom. Carefully examine the bottom of the tube before considering the reaction negative. (2) A "browning" of the medium is a negative reaction unless a black deposit is present. "Browning" occurs with TDA positive organisms.
URE	18-24 hr 36-48 hr	Red or Orange Red	Yellow Yellow or Orange	A method of lower sensitivity has been chosen. *Klebsiella*, *Proteus* and *Yersinia* routinely give positive reactions.
TDA	Add 1 drop 10% Ferric chloride.	Brown-Red	Yellow	(1) Immediate reaction. (2) Indole positive organisms may produce a golden orange colour due to indole production. This is a negative reaction.
IND	Add 1 drop Kovacs Reagent	Red Ring	Yellow	(1) The reaction should read within 2 minutes after the addition of the Kovacs reagents and the results recorded. (2) After several minutes, the HCl present in Kovacs reagent may react with the plastic of the cupule resulting in a change from a negative (yellow) colour to a brownish-red. This is a negative reaction.

SUMMARY OF RESULTS - 18-24 HOUR PROCEDURE (cont'd)

TUBE	INCUBATION	POSITIVE	NEGATIVE	COMMENTS
VP	Add 1 drop of 40% Potassium Hydroxide, then 1 drop of alpha-napthol.	Red	Colourless	(1) Wait 10 minutes before considering the reaction negative. (2) A pale pink colour which appears immediately after the addition of reagents but which turns dark `pink or red after 10 minutes should be interpreted as positive. Motility may be observed by hanging drop or wet mount preparation.
GEL		Diffusion of the pigment	No diffusion	(1) The solid gelatin particles may spread throughout the tube after inoculation. Unless diffusion occurs, the reaction is negative. (2) Any degree of diffusion is a positive reaction.
GLU		Yellow Or Gray	Blue or Blue-Green	COMMENTS FOR ALL CARBOHYDRATES Fermentation (*Enterobacteriaceae, Aeromonas, Vibrio*) (1) Fermentation of the carbohydrates begins in the most anaerobic portion (bottom) of the tube. Therefore, these reactions should be read from the bottom of the tube to the top. (2) A yellow colour at the bottom of the tube only indicates a weak or delayed positive reaction.
MAN INO SOR RHA SAC MEL AMY ARA		Yellow	Blue or Blue-Green	Oxidation (Other Gram-negatives) (1) Oxidative utilization of the carbohydrates begins in the most aerobic portion (top) of the tube. Therefore, these reactions should be read from the top to the bottom of the tube. (2) A yellow colour in the upper portion of the tube and blue in the bottom of the tube indicate oxidative utilization of the sugar. This reaction should be considered positive only for non-*Enterobacteriaceae* gram negative rods. This is a negative reaction for fermentative organisms such as *Enterobacteriaceae.*

SUMMARY OF RESULTS - 18- 24 HOUR PROCEDURE (cont'd)

TUBE	INCUBATION	POSITIVE	NEGATIVE	COMMENTS
GLU	After reading GLU reaction, add 2 drops 0.8% sulfanilic acid and 2 drops 0.5% N. N-dimethyl-alpha-naphthylamine			(1) Before addition of reagents, observe GLU tube (positive or negative) for bubbles. Bubbles are indicative of reduction of nitrate to the nitrogenous (N_2) state. (2) A positive reaction may take 2-3 minutes for the red colour to appear. (3) Confirm a negative test by adding zinc dust or 20 mesh granular zinc. A pink-orange colour after 10 minutes confirms a negative reaction. A yellow colour indicates reduction of nitrates to the nitrogenous (N_2) state.
	NO_2	Red	Yellow	
	N_2 gas	Bubbles: Yellow after reagents and zinc	Orange after reagents and zinc	
MAN INO SOR Catalase	After reading carbohydrate reaction, add 1 drop 1.5% H_2O_2	Bubbles	No bubbles	(1) Bubbles may take 1-2 minutes to appear. (2) Best results will be obtained if the test is run in tubes which have no gas from fermentation.

IDENTIFICATION OF NON-ENTERIC GRAM-NEGATIVE RODS (API 20NE)

Principle

The API 20NE system facilitates the identification of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae* within 48 hours.

The API 20NE strip consists of microtubes containing dehydrated media and substrates. The media microtubes containing conventional tests are inoculated with a bacterial suspension which reconstitutes the media. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. The substrate microtubes contain assimilation tests and are inoculated with a minimal medium. If the bacteria are capable of utilizing the corresponding substrate, then they will grow.

Materials

API 20NE strips - store at 2-8^{0}C
0.85% sterile saline
Mineral oil
Zinc dust
AUX Medium }
James Reagent }
Nitrate 1 - store at 2-8^{0}C } Store at 2-8^{0}C
Nitrate 2 - store at 2-8^{0}C }
Oxidase Reagent

Procedure

1. Preparation of Inoculum

   a) Add 2 ml. of 0.85% saline to a sterile test tube.

   b) Using a sterile inoculating loop, carefully touch the centre of a well isolated colony (2-3 mm. Diameter) and thoroughly emulsify in the saline. The suspension turbidity should be equal to a 0.5 McFarland standard.

2. Preparation of the Strip

   a) An incubation tray and lid are supplied for each strip.
   b) Dispense 5 ml of distilled water in to the tray.

3. Inoculation of the Strip

a) Remove the cap from the tube containing the bacterial suspension and insert a sterile pipette.
b) Tilt the API 20NE incubation tray and fill the TUBE section of the NO_3 to PNPG microtubes by placing the pipette tip against the side of the cupule.
c) Open an ampule of AUX Medium and add 200 uL of the bacterial suspension to the ampule. Mix well with a pipette while avoiding the formation of air bubbles.
d) Using the AUX Medium bacterial suspension, fill both the TUBE and CUPULE section of [GLU] to [PAC]. Do not overfill the cupules. Fill to a flat or slightly convex meniscus.
e) After inoculation, completely fill the CUPULE section of the 3 underlined tests, GLU, ADH and URE tubes with mineral oil.
f) Using the excess bacterial suspension, inoculate an agar slant or plate (non-selective media such as nutrient agar, blood agar or tryptic (trypticase) soy agar is suggested) as a purity check and for oxidase testing, and/or additional biochemical testing. Incubate the slant or plate with the API 20NE strip.

4. Incubation of the Strip

a) After inoculation, place the plastic lid on the tray and incubate the strip for 24 hours at 30^0C in a non-CO_2 incubator.

5. Reading the Strip

a) After 24 hours incubation, record all reactions not requiring the addition of reagents.
b) Perform the oxidase test.

A portion of the growth from the agar slate or plate, inoculated from the 20NE bacterial suspension, should be rubbed onto filter paper to which a drop of oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) has been added. The area where the growth has been added will turn dark purple within 10 seconds if the reaction is positive and will be colourless or light purple if negative.

Note: (a) Nichrome wire loops should NOT be used in performing the oxidase test. Nichrome wire can cause a false positivereaction.

(b) The oxidase test should NOT be performed using bacterial growth from selective media such as MacConkey, EMB, etc.

c) Assimilation tests are observed for bacterial growth. An opaque cupule indicates a positive reaction.
d) Protect the assimilation tests with the incubation tray lid during the reading of the Nitrate and TRP tests.
e) Perform the Nitrate test.

   i. Add one drop of Nitrate 1 and one drop of Nitrate 2 reagents to NO_3 cupule.
   ii. After 5 minutes a red color indicates a positive reaction.
   iii. A negative reaction may be due to the production of nitrogen. Add Zinc dust to the NO_3 cupule. After 5 minutes a colorless cupule indicates a positive reaction. A pink-red cupule indicates a negative reaction.

f) Perform the TRP test.

   i. Add one drop of JAMES Reagent.
   ii. The reaction takes place immediately, producing a pink color in the entire cupule if the reaction is positive.

Interpretation

1. Use the API 20NE analytical profile index.

2. The tests are separated into groups of three. The following numerical value is assigned to each positive reaction recorded:

   1 - positive reaction in the first test of the group
   2 - positive reaction in the second test of the group
   4 - positive reaction in the third test of the group

   By adding the values corresponding to positive reactions in each group, a seven digit number is obtained.

3. The strip must be reincubated in the following cases:

   i. If the profile cannot be found in the Analytical Profile Index.
   ii. If the following note is indicated for the profile obtained:

   IDENTIFICATION NOT VALID
   BEFORE 48-HR INCUBATION

   iii. If the strip is to be reincubated, remove the reagents from the NO_3 and TRP cupules and then cover these tests with mineral oil.

iv. Reincubate the strip for another 24 hours at 30^0C in a non-CO_2 incubator.
v. Read all the tests again, except for NO_3, TRP and GLU.

READING TABLE

TESTS	SUBSTRATES	REACTONS/ENZYMES	NEGATIVE RESULTS	POSITIVE RESULTS
$N0_3$	Potassium nitrate	NITrate reduction to nitrites	**NIT 1 + NIT 2 / 5 min** colourless	pink-red
		NITrates to nitrogen	**Zn / 5 min** pink	colourless
TRP	tryptophane	indole production	**JAMES / immediate** colourless / pale green / yellow	pink
GLU	glucose	Acidification	blue to green	yellow
ADH	arginine	arginine dihydrolase	yellow	orange/pink/red
URE	urea	Urease	yellow	orange/pink/red
ESC	esculin	hydrolysis (β-glucosidase)	yellow	grey/brown/black
GEL	gelatine (with India ink)	hydrolysis (protease)	no pigment diffusion	diffusion of black pigment
PNPG	p-nitrophenyl-β-D-galactopyranoside	β-galactosidase	colourless	yellow
[GLU]	glucose	Assimilation	transparent	opaque
[ARA]	arabinose	Assimilation	transparent	opaque
[MNE]	mannose	Assimilation	transparent	opaque
[MAN]	mannitol	Assimilation	transparent	opaque
[NAG]	N-acetyl-glucosamine	Assimilation	transparent	opaque
[MAL]	maltose	Assimilation	transparent	opaque
[GNT]	gluconate	Assimilation	transparent	opaque
[CAP]	caprate	Assimilation	transparent	opaque
[ADI]	adipate	Assimilation	transparent	opaque
[MLT]	malate	Assimilation	transparent	opaque
[CIT]	citrate	Assimilation	transparent	opaque
[PAC]	phenyl-acetate	Assimilation	transparent	opaque
OX	see oxidase test	cytochrome oxidase	colorless/ light purple	dark purple

SYSTEM FOR IDENTIFICATION OF *NEISSERIA* & *HAEMOPHILUS* (API NH)

Principle

The API NH strip consists of 10 microtubes containing dehydrated substrates, which enable the performance of 12 identification tests (enzymatic reactions or sugar fermentations), as well as the detection of a penicillinase (particular interest in *Haemophilus influenzae, Haemophilus parainfluenzae, Branhamella catarrhalis (Moraxella catarrhalis)* and *Neisseria gonorrhoeae).*

The reactions produced during incubation result in spontaneous color changes or are revealed by the addition of reagents.

After a 2-hour incubation period at a temperature of 35-37^{o}C, the reading of the reactions is performed visually and identification is obtained by consulting the profile list.

Reagents

API NH strips
NaCl 0.85% Medium (2 ml)
JAMES reagent
ZYM B reagent
Swab
Incubation box
Result sheet
1 package insert
McFarland Standard, point 4 on the scale
Mineral oil
Pipettes
Ampule rack
Ampule protector

Procedure

1. Specimen Processing

The microorganisms to be identified must first be isolated as separate colonies by streaking the specimen onto Blood agar, Chocolate agar or Martin-Lewis agar according to standard

microbial techniques.

2. Preparation of Strip

Each strip is composed of 10 cupules. Each cupule has an open and closed area (cupule and tube). An incubation tray is supplied for each strip. It serves as a support and individual chamber while both protecting the strip from contaminants in the air and assuring the humid atmosphere necessary to avoid dehydration during incubation.

- Remove the strip from its individual packaging
- Place the strip in the incubation box
- Discard the desiccant sachet

Record the specimen number on the flat portion of the tray (do not record the number on the lid as it may be misplaced during handling).

3. Preparation of the Inoculum

- Open an ampule of NaCl 0.85% Medium (2 ml) with the ampule protector.
- Using a swab, pick up a few well-isolated colonies and prepare a suspension with a turbidity equivalent to **4 McFarland, ensuring it is well mixed**.
- The suspension should be used immediately after preparation.

4. Inoculation of the Strip

- Distribute the prepared bacterial suspension into the cupules, avoiding the formation of bubbles (tilt the strip slightly forwards and place the tip of the pipette or PSIpette against the side of the cupule):
  - Only fill the tube part of the first 7 microtubes (PEN to URE): about 50 µl.
  - Fill tube and cupule of the last 3 microtubes LIP/ProA, PAL/GGT, βGAL/IND: about 150 µl, avoiding the formation of a convex meniscus.
- Cover the first 7 tests (PEN to URE) with mineral oil (underlined tests).

NOTE: The quality of the filling is very important: tubes which are insufficiently or excessively full may cause false positive or false negative results.

- Close the incubation box.
- Incubate for 2 hours at 35-37^{o}C **in aerobic conditions.**

5. Incubation

Incubate for 2 hours at 35-37°C in aerobic conditions.

6. Reading the Strip

Refer to the Reactions Table for a description of how to read the reactions.

Note all spontaneous reactions (PEN to βGAL) and record them as + or -.

- Add 1 drop of ZYM B reagent to microtubes 8 and 9: LIP/ProA and PAL/GGT.
- Add 1 drop of JAMES reagent to microtube 10: βGAL/IND.
- **Wait 2 minutes** then read the reactions by referring to the Reading Table in this package insert and record them on the result sheet.
  - If the LIP reaction is positive (blue pigment), interpret the ProA reaction as **negative**, whether the ZYM B reagent has been added or not.
  - If, after a 2-hour incubation period, several reactions (fermentation, penicillinase) are doubtful, re-incubate the strip for another 2 hours and read the reactions again (the enzymatic tests should not be re-read in this case).

Reactions Table

TESTS	REACTIONS	SUBSTRATES	QTY (mg)	RESULTS	
				NEGATIVE	POSITIVE
1) PEN	PENicillinase	Penicillin G	1.36	Blue (penicillinase absent)	Yellow Yellow-green Yellow-blue (penicillinase present
2) GLU 3) FRU 4) MAL 5) SAC	GLUcose (Acidification) FRUctose (Acidification) MALtose (Acidification) SACcharose/Sucrose (Acidification)	Glucose Fructose Maltose Sucrose	0.5 0.1 0.1 0.5	Red Red-orange	Yellow Orange
6) ODC	Ornithine DeCarboxylase	Ornithine	0.55	Yellow-green Grey-green	Blue
7) URE	UREase	Urea	0.41	Yellow	Pink-violet
8a) LIP	LIPase	5-bromo-3-indoxyl-caprate	0.033	Colorless Pale grey	Blue (+precipitate)
9a) PAL	Alkaline Phosphatase	Para-Nitrophenyl-phosphate 2CHA	0.038	Colorless Pale yellow	Yellow
10a) βGAL	Beta GALactosidaase	Para-Nitrophenyl-BD galactopyranoside	0.04	Colorless	Yellow

Reactions Table (Cont'd)

TESTS	REACTIONS	SUBSTRATES	QTY (mg)	RESULTS	
				NEGATIVE	POSITIVE
8b) ProA	Proline Arylamidase If LIP is +. ProA is always -	Proline-4-methoxy- β naphthylamide	0.056	ZYM B / 3 min Yellow Pale orange (brown if LIP +)	ZYM B / 3 min Orange
9b) GGT	Gamma Glutamyl Transferase	Gamma glutamyl 4-methoxy- β naphthylamide	0.049	ZYM B / 3 min Yellow Pale orange (yellow-orange if PAL +)	ZYM B / 3 min Orange
10b) IND	INDole	Tryptophane	0.036	JAMES / 3 min Colorless	JAMES / 3 min Pink

Quality Control

To be performed on receipt of every new lot of strip by the Q.C bench technologist.

Reference

QC organisms to be used:

Neisseria gonorrhoea	ATCC 31426
Haemophilus influenzae	ATCC 10211
Branhamella catarrhalis	ATCC 23246
Haemophilus paraphrophilus	ATCC 49917

Reference Package Insert - api NH system for the identification of *Neisseria* and *Haemophilus* bioMerieux Inc., Missouri USA.

API VOICE RESPONSE

1. Dial (800) 645-7056 from a touch tone phone.

2. Enter product code access number (0-14).

3. Press * symbol.

4. Enter profile number as outlined below.

5. Press # symbol.

6. Press 1# to end the call.
   Press 2# to repeat the identification.
   Press 3# for the next profile.
   Press 4# to speak to a technologist.

ACCESS CODES

Product Code Access Number		Incubation Time	Profile Number Format
0	API 20E	24 hours	Enter 7 digits
1	API 20E	48 hours	Enter 9 digits only
6	API 20C	48-72 hours	Enter 7 digits
7	Coryne	24 hours	Enter 7 digits
9	STAPH-IDENT	24 hours	Enter 7 digits
12	API NE (Rapid NE)	24/48 hours	Enter 7 digits
35	20 Strep	24 hours	Enter 7 digits

BACITRACIN DISK TEST

Principle

This is a screening test for the presumptive identification of Group A Streptococci which are susceptible to 0.04U bacitracin. Other beta-haemolytic streptococci are usually resistant to this concentration of bacitracin.

Reagents

Bacto Differentiation Disks Bacitracin (0.04U). Store refrigerated.
Blood Agar (BA)

Other Materials

Culture loop
Cotton swabs
Forceps

Procedure

1. Inoculate the surface of the BA with the suspect beta haemolytic *Streptococcus*. Streak for confluent growth.
2. Using aseptic technique, place a bacitracin disk onto the inoculated surface.
3. Incubate in O_2 at 35^{o}C X 18-24 hr.

Interpretation

Susceptible: any zone of inhibition around the disk (Presumptive Group A Streptococcus).
Resistant: growth up to the edge of the disk

Precautions

1. Other beta-haemolytic streptococci may be susceptible to bacitracin. Therefore this test can be used only for the presumptive identification of Gp. A Strep.

Quality Control

Test with known susceptible and resistant control strains weekly.

Susceptible: Gp.A Strep. (ATCC 19615)
Resistant: Gp.B Strep. (ATCC 13813)

Reference

1. Difco Differentiation Disks Bacitracin package insert.

BETA-LACTAMASE (CEFINASE) TEST

Principle

Cefinase discs are intended for use in rapid testing of isolated colonies of *Neisseria gonorrhoeae, Staphylococcus* species, *Enterococcus* species, *Hameophilus influenzae* and anaerobic bacteria for the production of beta-lactamase.

The Cefinase disc is impregnated with the chromogenic cephalosporin, Nitrocefin. This compound exhibits a very rapid colour change from yellow to red as the amide bond in the beta lactam ring is hydrolyzed by a beta-lactamase. When a bacterium produces this enzyme in significant quantities, the yellow-colored disc turns red in the area where the isolate is smeared.

Although other penicillins and cephalosporins may be used as substrates for specific enzymes, Nitrocefin has the wide spectrum of susceptibility and sensitivity of the commercially available beta lactams. It is not known to react with other microbial enzymes.

Each disc is used to test one bacterial strain for the presence of beta-lactamase.

Materials

Cefinase discs impregnated with Nitrocefin.

Procedure

1) Using a single disk dispenser, dispense the required number of disks from the cartridge into an empty petri dish or onto a microscope slide.

2) Moisten each disc with 1 drop of Sterile distilled water.

3) With a sterilized loop or applicator stick remove several well-isolated similar colonies and smear onto a disk surface.

4) Observe disk for colour change.

5) Alternate procedure: Using forceps moisten disk with one drop of Purified Water and then wipe across colony.

Interpretation

A positive reaction will show a yellow to red colour change on the area where the culture was applied. Note: colour change does not usually develop over the entire disk. A negative result will show no colour change on the disc.

For most bacterial strains a positive result will develop within 5 minutes. However, positive reactions for some staphylococci may take up to 1 hour to develop.

Organisms	Result	Approx. Reaction Time	Interpretation
Staphylococcus aureus	Positive	1 hr	Resistant to penicillin, ampicillin, carbenicillin. Probably susceptible to cephalothin, methicillin, oxacillin, naficillin and other penicillinase-resistant penicillins.
Enterococcus faecalis	Positive	5 min	Resistant to penicillin and ampicillin.
Hameophilus influenzae	Positive	1 min	Resistant to ampicillin Susceptible to cephalosporins.
Neisseria gonorrhoeae and *Branhamella catarrhalis*	Positive	1 min	Resistant to penicillin.
Anaerobic bacteria	Positive	30 mins	Probable identification is *Bacteroides* species. Probably resistant to penicillin and may be resistant to cephalosporins including cefotaxime and rarely cefoxitin.

Controls: *Staphylococcus aureus* (ATCC 29213): Positive
Haemophilus influenzae (ATCC 10211): Negative

Reference

1. Murray P.A., et al. Manual of Clinical Microbiology, 7th ed. 1999.

BILE ESCULIN TEST

Principle

This test determines the ability of an organism to grow in the presence of bile and to hydrolyze the glycoside esculin to esculetin and glucose. The test is used to presumptively identify Group D Streptococci.

Materials

Bile esculin agar slant / plate
Culture loop

Procedure

1. Heavily inoculate a bile esculin slant / plate with the suspect organism.
2. Incubate in O_2 at 35^{o}C for 18-24 hr.

Interpretation

Positive: Presence of a dark brown to black colour on the slant.

Negative: No blackening of the medium. Growth may occur, but this does not indicate esculin splitting.

Quality Control

Each new lot of media should be tested with known control strains.

Positive:	*E. faecalis*	(ATCC 29212)
Negative:	Gp.B Strep.	(ATCC 13813)
No Growth:	Gp.A Strep.	(ATCC 19615)

References

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, p4-12.

BILE SOLUBILITY TEST

Principle

Tests the ability of alpha haemolytic streptococci to lyse in the presence of bile salts. This test is used for the identification of *Streptococcus pneumoniae*.

Reagents

BBL Spot Test dropper (10% sodium desoxycholate).

Procedure

1. Hold the dropper upright and squeeze gently to crush the glass ampoule inside the dispenser.
2. Place 1 drop of the reagent directly on isolated colonies of suspected *S. pneumoniae*.
3. Keep the plates very level to prevent the reagent from running and washing a non-pneumucoccal colony away, producing a false positive result.
4. Incubate at room temperature on the bench for 15-30 minutes until the reagent drys. Do not invert the plate; leave the lid ajar.
5. Examine the colonies for lysis.

Interpretation

Positive (bile soluble): Lysis of the colonies.

Negative (bile insoluble): No lysis of colonies.

Quality Control

Test with known positive and negative control strains weekly.

Positive: *S. pneumoniae* (ATCC 6303)
Negative: Viridans Streptococcus (LPTP 8610)

References

1. Murray PA, et al. Manual of Clinical Microbiology, 7th ed., 1999; p. 1665.

2. BBL Desoxycholate Reagent Droppers package insert, April 1991.

CATALASE TEST

Principle

Detects the presence of the enzyme catalase which hydrolyzes H_2O_2 to produce H_2O and O_2. This test is used to differentiate Staphylococci (catalase positive) from Streptococci (catalase negative).

Reagents

Hydrogen peroxide (H_2O_2), 3%

Store in a dark bottle and avoid any undue exposure to light.
Keep refrigerated at all times when not in use.

Other Materials

Clean glass microscope slides
Plastic culture loop or wooden applicator stick

Procedure

1. Pick a colony from an 18-24 hr culture and place it on a clean glass slide. Avoid carry over of blood agar which can cause false positives.
2. Put one drop of 3% H_2O_2 over the organism on the slide. Do not reverse the order of the procedure as false positive results may occur. Do not mix.
3. Observe for immediate bubbling (gas liberation) and record the result.
4. Discard the slide into a discard container.

Interpretation

Positive test: Immediate bubbling, easily observed (0_2 formed)

Negative test: No bubbling

Precautions

1. Carry over of blood agar must be avoided.
2. Growth for testing must be from an 18-24 hr culture.
3. 3% H_2O_2 is caustic - avoid exposure to skin. If H_2O_2 does get on the skin, immediately flood the area with 70% ethyl alcohol, not water.

4. Aerosols may be released by the bubbling of the O_2.
5. H_2O_2 is unstable and breaks down easily on exposure to light. The solution must be kept refrigerated in the dark.

Quality Control

H_2O_2 is very unstable and should be tested daily or immediately prior to its use.

Positive: *S. aureus* (ATCC 25923)
Negative: Gp. A. Strep. (ATCC 19615)

References

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD., 1980, p51-58.

2. Murray PA, et al. Manual of Clinical Microbiology, 7th ed., 1999; pp 426-427.

CETRIMIDE PSEUDOMONAS SELECTIVE AGAR

Principle

Cetrimide Selective Agar is used for the identification of *Pseudomonas aeruginosa.* Cetrimide is a compound that has germicidal activity against most organisms except *Pseudomonas aeruginosa*. Also pigment production is enhanced on this media.

Procedure

1. Divide the plate into approximately 8 pie shaped divisions.
2. Streak the test organism (pure culture) onto one of the pie shaped divisions.
3. Incubate at 35^0C for 18 - 24 hours.

Interpretation

Pseudomonas aeruginosa will grow on this media and will be pigmented a pale green to dark blue-green colour. All other organisms will not grow or will be non-pigmented.

Quality Control

Test with positive and negative controls each time the test is set up.

Positive: *Pseudomonas aeruginosa* (ATCC 27853)
Negative: *Escherichia coli* (ATCC 25922)

Reference

1. PML Technical Manual, 1990.

CRYPTOCOCCAL ANTIGEN

Latex particles coated with anti-cryptococcal globulin (ACGR) reacts with cryptococcal polysaccharide antigen (in CSF or serum) causing a visible agglutination.

I. Specimen Collection and Processing

5 mL of blood is collected in a serum separator tube and separated by centrifugation. The serum is removed to a vial and refrigerated until testing. Specimens are stored a -70^{o}C after testing.

Spinal fluid is collected in clean, sterile, centrifuge tubes. Specimens are stored refrigerated after testing.
Note: Fungus culture should also be set up.

II. Procedure

Reagents

Meridian CALAS (Cryptococcal Antigen Latex Agglutination System)

1. GBDA - Glycine buffered diluent with albumin.
2. ACGR - Anti-cryptococcal globulin reagent.
3. NGR - Normal globulin reagent.
4. AGC - Antiglobulin control. Rehydrate with 1.5 mL dH_2O.
5. NC - Negative control. Rehydrate with 2.5 mL dH_2O and **inactivate the negative at 56^{o}C for 30 minutes**.
6. CAC - Cryptococcal antigen control (Positive control).
7. Pronase - Rehydrate with 2.5 mL dH_2O.

Note: Ensure that all reconstituted vials are thoroughly dissolved before use

All reagents are stored refrigerated. Do not interchange reagents with a kit having a different lot number. Allow reagents to warm to room temperature before use. Mix gently before use.

Other Materials
Boiling water bath
56°C heating block
1.0 x 0.1 mL pipettes
Rotator
Small serologic test tubes
Test tube rack
Marking pen
Applicator sticks

The following are provided by Meridian:
Capillary pipettes
Rubber bulb
Ring slide

Method

Specimen preparation:

1. Store refrigerated if testing is not done immediately.

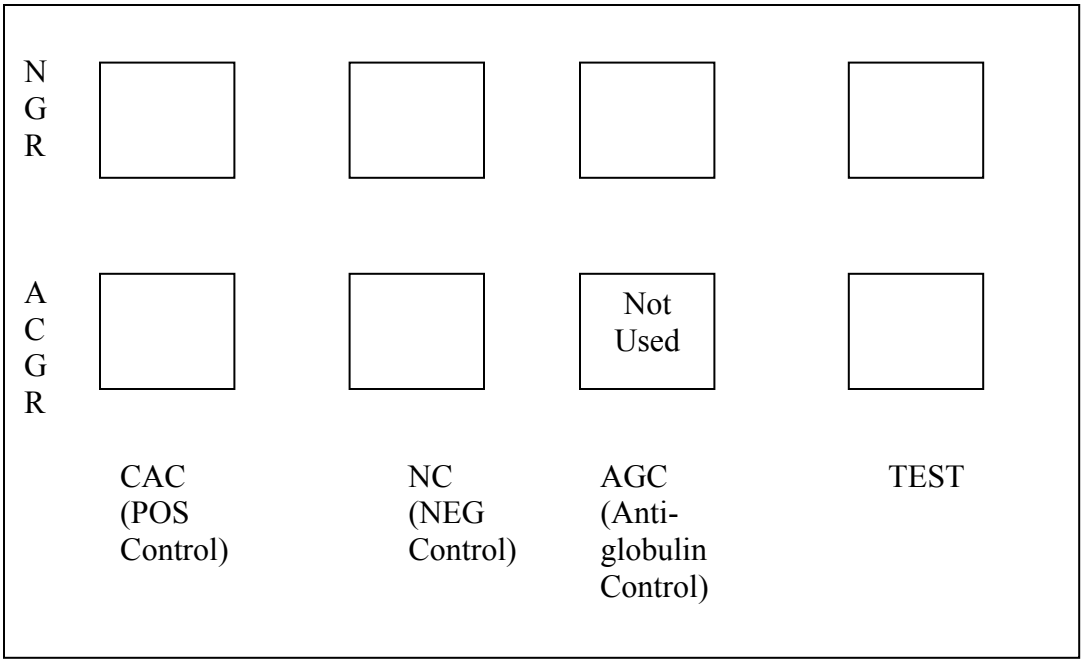
    (a) Inactivate serum by mixing 500 µL of serum and 500 µL of pronase solution in a 12 x 75 mm tube and incubate at 56°C for 15 minutes. Further inactivate in a boiling water bath for 5 minutes. **This constitutes a 1:2 dilution.**

    (b) Centrifuge CSF at 3500 rpm for 15 mins. Inactivate the supernatant in a boiling water bath for 5 minutes.

Performing the tests:

Note: Controls must be run each time a patient specimen is tested.

1. Set up and label the slide as follows:



2. Gently resuspend the latex particles in the ACGR and NGR reagents. Rock each reagent just prior to use.
   Place one drop of ACGR or NGR into the designated rings.

3. Place 25 µL (one drop) of the cryptococcal antigen control (CAC) into the designated rings. Repeat with the negative control (NC) and anti-globulin control (AGC)

4. Place 25 µL of specimen in the designated rings.

5. Using a separate applicator stick, mix the contents of each ring thoroughly, spreading the contents over the entire surface area.

6. Place the slide on the rotator and rotate at 125 rpm for 5 minutes.

7. Read the reactions immediately.

8. Rate the agglutination as follows:
   Positive = any evidence of agglutination (granulation or clumping)
   Negative = a homogenous suspension of particles with no visible clumping.

9. Patient specimens showing any agglutination in ACGR should be titrated against both ACGR and NGR reagents.

   (a) Prepare two-fold serial dilutions of the specimen using 200 µL of GBDA in each of 8 test tubes labelled as follows:

Tube 1 2 3 4 5 6 7 8

Serum 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512

CSF 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256

   (b) Transfer one drop of each dilution into 2 rings.
   (c) Add one drop of ACGR to one ring of each dilution.
   (d) Add one drop of NGR to each of the other rings.
   (e) Mix using separate applicator sticks.
   (f) Place the slide on the rotator and rotate at 125 rpm for 5 minutes.
   (g) Read the results as follows:

      1+ = fine granulation against a milky background
      2+ = small but definite clumps against a slightly cloudy background
      3+ = large and small clumps against a clear background
      4+ = large clumps against a clear background

   (h) If tube #8 gives an agglutination of 2+ or greater, the specimen must be further serially diluted until a titre may be obtained.

Interpretation of results

Negative: Negative result in initial screening tests against ACGR.

Positive: The titre is reported as the highest dilution showing a 2+ or greater reaction with ACGR and negative with NGR.

Nonspecific Interference: The titre with ACGR is at least 4-fold higher than the titre with NGR.

Uninterpretable test: The titre with ACGR is less than 4-fold greater than the titre with NGR.

III. Reporting

Telephone all positive reports.

Negative Report: "Cryptococcal antigen not detected by latex agglutination."

Positive Report: "Cryptococcal antigen detected at a titre of _____ by latex agglutination."

Non-specific or Uninterpretable Report:
"Cryptococcal antigen uninterpretable by latex agglutination."

IV. Precautions

The ring slide must be thoroughly cleaned after each use as follows:

(a) Soak in hypochlorite overnight.
(b) Scrub using detergent.
(c) Rinse well with tap water.
(d) Rinse 3 times with distilled water.
(e) Dry thoroughly using paper towels.
(f) Wipe clean with lint-free tissue.

V. Quality Control

The pattern of reactions for the controls must be as follows.

NGR	-	-	+
ACGR	+	-	
	CAC	NC	AGC

Failure to obtain this pattern indicates that the test must be repeated and the patient test results cannot be reported.

VI. References

Product Insert, 1986. Meridian Diagnostics Inc., 3471 River Hills Dr., Cincinnati, Ohio 45244. (513)-271-3700.

CRYSTAL MRSA IDENTIFICATION SYSTEM

Principle

Used as a screening test for the detection of intrinsic methicillin-resistant *Staphylococcus aureus* from isolated colonies.

Materials

BBL Crystal panel (lid and base)
MRSA Id both-3.2ml
transfer pipette
(all provided in kit)

Method

1. Suspend test *S. aureus* in 2 ml of Vitek saline and adjust to a McFarland 0.5.
2. Vortex and transfer 0.5mL to the tube of MRSA Id broth and Vortex.
3. Remove lid from panel base without touching lid prongs and discard desiccant.
4. Place a drop of sterile saline in the first well (positive control).
5. Using the same pipette, place 3 drops of the ID broth suspension into the same well.
6. Place 4 drops of the broth suspension into the next 2 wells of the panel (oxacillin and negative control). Leave the fourth well empty. Remove any bubbles.
7. Cover the panel base with the lid. Gently press lid onto panel base with the lid onto panel base with a snap. Lid should no longer be removed. Do not invert panel.
8. Incubate at 35^{o}C for at least 4 but not more than 5 hours.
9. Expose panel to UV light and record which wells are fluorescing.

Interpretation

Bacteria	Well#1	Well#2	Well#3
Methicillin Sensitive:	+	–	–
Methicillin Resistant:	+	+	-

Quality Control

Results are uninterpretable if positive control well is negative or the negative control well is positive.

Reference

1. BBL crystal MRSA ID System package insert August 1993.

DENKA MRSA SCREEN

Principle

To be used as a screening test for the detection of Methicillin Resistant *S. aureus* (MRSA) from isolated colonies.

Reagents

MRSA screening kit
Microcentrifuge tubes
Boiling water bath
Wooden sticks
Loops
Micropipettes

Method

1. Add 4 drops of extraction reagent #1 into a microcentrifuge tube.

2. Take one heavy loopful of the *Staphylococcus aureus* colonies from a blood plate and suspend the cells in the microcentrifuge tube.

3. Place in a boiling water bath for 3 minutes.

4. Remove microcentrifuge tube and let cool to room temperature.

5. Add one drop of extraction reagent #2 to the tube and mix well.

6. Centrifuge at high speed for 5 minutes.

7. For each specimen to be tested, allot and label one circle of the test card for testing with sensitized latex and one with control latex.

8. Place 50 microliters of the specimen onto 2 of the test circles and add one drop of the sensitized cells to one circle and one drop of the latex control to the other.

9. Mix the sample and latex together.

10. Rotate the card by hand for 3 minutes and observe for agglutination.

11. If negative rotate for another 3 minutes.

Interpretation

Sensitized latex	Control latex	Result
+	-	MRSA
-	-	Not MRSA
+ or -	+	Indeterminant

Quality Control

Positive and negative controls must be set up once per week.

Positive: *S. aureus* (ATCC 43330)
Negative: *S. aureus* (ATCC 29213)

Reference

1. Denka Seiken Co., Ltd., Tokyo, Japan, Denka MRSA Screen package insert June 1998.

DNAse TEST

Principle

This test determines the ability of an organism to produce deoxyribonuclease (DNAse). This test used, in conjunction with others, for the identification of *S. aureus*, *M. catarrhalis* and *Serratia* species.

Reagents

DNAse test agar with methyl green

Other Materials

Culture loop or wooden applicator stick

Procedure

1. Spot inoculate an isolate using growth from an 18-24 hr pure culture.
2. Incubate in O_2 at 35^{o}C X 18-24 hr.

Interpretation

The plate should be observed against a white background.

Positive: Distinct clear zone surrounding spot inoculum
Negative: No clear zone

Quality Control

Test each new batch of media and when in use with positive and negative controls.

Positive: *S. aureus* (ATCC 25923)
Negative: *S. epidermidis* (ATCC 12228)

Reference

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD., 1980, p94-113.

***E. coli* O157 LATEX TEST (OXOID)**

Principle

The Latex test will demonstrate by slide agglutination, *E. coli* strains possessing the O157 antigen. Sorbitol MacConkey Agar (SMAC) should be used as the primary screen. Non-sorbital fermenting colonies (NSF) are tested with the latex reagents, to determine if the isolate belongs to the O157 serogroup, and is therefore a potential vero-cytotoxin (VT) producing strain.

Reagents

DR621 Test Latex - consists of latex particles sensitized with specific rabbit antibody reactive with the O157 antigen.

DR622 Control Latex - consists of latex particles sensitized with pre-immune rabbit globulins.

Storage

Do not freeze. Store at 2^0C - 8^0C. Do not use kit beyond the expiry date.

Procedure

NSF colonies may be taken from SMAC plates or alternatively NSF isolates may be inoculated onto non-selective agar media for testing.

It is necessary to test up to 10 separate NSF colonies to ensure a high probability of detection from mixed cultures.

1) Bring the latex reagents to room temperature. Make sure the latex suspensions are mixed by vigorous shaking. Expel any latex from the dropper pipette for complete mixing.

2) Dispense 1 drop of the Test latex onto a circle of the black slide. Place it close to the edge of the circle.

3) Add some loopfuls or a pasteur pipette drop of saline to the circle. Ensure that the latex and saline do not mix at this stage.

4) Using a loop, pick off a portion of the colony to be tested and carefully emulsify in the saline drop.

5) Mix the Test latex and suspension together and spread to cover most of the reaction area using the loop. Flame the loop.

6) Rock the slide in a circular motion, observing for agglutination. Do not rock the card for more than 1 minute and do not use a magnifying glass.

7) If no agglutination occurs, then proceed to test other NSF colonies if these are present.

8) If agglutination with the test reagent does occur, then it is necessary to test a further portion of the colony with the control reagent to ensure that the isolate is not an autoagglutinating strain.

9) When finished, dispose of the reaction slide into disinfectant.

Interpretation

a) Positive result - Agglutination of the Test latex occurs within 1 minute. No agglutination of the Control latex. *Perform biochemical tests to confirm that the organism is an *E. coli* strain.

b) Negative result - no agglutination of the Test latex.

c) Non-interpretable result - clumping of the Control latex.

References

1. Borczyk A., Lior H., Crebin B. 1987. Int. J. Food. Microbiol. 4, 347-349.

2. Konowalchuk J., Speirs J. and Stavric S. 1977. Infect. Immune. 18, 775-779.

3. Scotland S., Day N. and Rowe B. 1980. FEMS Microbiol. Lett. 7, 15-17.

4. Centers for Disease Control. 1982. Morbid Mortal Wkly. 31, 580-585.

5. Karmali M., Steel B., Petric M. and Lim C. 1983. Lancet 8, 619-620.

6. Johnson W., Lior H. and Bezanson. 1983. Lancet 8, 76.

7. March S. and Ratnam. 1986. J. Clin. Microbiol. 23, 869-872.

8. Krishnan C., Fitzgerald V., Dakin S. and Behme R. 1987. J. Clin. Microbiol. 25, 1043-1047.

GERM TUBE TEST

Principle

This is a rapid test for the presumptive identification of *C. albicans.*

Reagents

Bovine serum

A small volume to be used as a working solution may be stored at 2 to 8°C. Stock solution can be dispensed into small tubes and stored at -20°C.

Other Materials

Clean glass microscope slides
Glass coverslips
Vitek tubes (13 x 100 mm)
Pasteur pipettes

Procedure

1. Put 3 drops of serum into a small Vitek tube.
2. Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum. The pipette can be left in the tube.
3. Incubate at 37°C for 2-4 hours but no longer.
4. Transfer a drop of the serum to a slide for examination.
5. Coverslip and examine microscopically using x 40 objective.

Interpretation

Germ tubes are appendages half the width and 3 to 4 times the length of the yeast cell from which they arise. There is no constriction between the yeast cell and the germination tube.

Positive test: presence of short lateral filaments (germ tubes)

Negative test: yeast cells only

Precaution

C. tropicalis may form pseudohyphae which may be falsely interpreted as germ tubes.

Quality Control

Set up known controls each time a test is run.

Positive:	*C. albicans*	(ATCC 10231)
Negative:	*C. tropicalis*	(ATCC 13803)

Reference

1. Murray PA, et al. Manual of Clinical Microbiology, 7th ed., 1999; pp. 1189-1191.

GONOGEN (GC COAGGLUTINATION) TEST

Principle

The Gonogen II test is a coagglutination test for the confirmatory identification of *N. gonorrhoeae*.

Reagents

I: Buffer
II: Gonogen reagent (antibodies)
Positive control reagent
Negative control reagent

Other Materials

Test tray: consists of wells with special matrix and absorbent material
Glass tubes (12 x 75mm) (not provided)
Glass dropper rod assembly
Plastic transfer pipets

Procedure

1. Preparation of sample

   a) In a 12x75 mm tube dispense 500 µL of reagent I (buffer) using the glass dropper rod assembly provided (demarcation line).

   b) Using a swab, make a suspension of approximately 30 colonies to match a McFarland 1 turbidity standard.

   c) Press swab against side of tube to express as much liquid as possible.

   d) Vortex reagent II and add 1 drop to the tube.

   e) Mix and set sit for at least 5 minutes.

2. Test

   a) With a plastic transfer pipet, add 2 drops of each test suspension into a well of the test tray using a separate well for each test.

   b) using a clean plastic transfer pipet, add 1 drop of reagent I (buffer) to each completed test well.

Interpretation

Positive: Pink to red dot in well of test tray.

Negative: White to pale pink dot in well of test tray.

Note: 1. A colour reaction more intense than the negative control should be interpreted as positive.

2. If color reaction is questionable, reincubate tube at RT for 3 minutes and repeat test.

3. If specimen suspension is made too turbid a faint background colour will occur. This should NOT be interpreted as a positive result.

Quality Control

The positive and negative controls must be tested whenever a test is run. The test is performed in the same manner except 1 drop of the control reagent is added to 500 µL of buffer rather than a suspension of the test organism.

References

1. Gonogen II package insert, October 1993.

HIGH LEVEL AMINOGLYCOSIDE TESTING

Principle

Enterococcal species where an identification and sensitivity has been performed must be tested for resistance to vancomycin and high level gentamicin and streptomycin (HLAR).

Materials

Entero HLAR Bi-plates
Brain Heart Infusion Agar (BHI) - Control Plates

Procedure

1. Using the VITEK colorimeter, prepare a 0.5 McFarland suspension in sterile saline (inoculum from VITEK can be used).

2. Using a sterile swab, spot inoculate the suspension onto each half of the plates. Three organisms can be tested on each plate.

3. After the inocula has dried, incubate the plate at 35^{o}C for up to 48 hours.

Interpretation

Check the control plate for adequate growth. Then check the drug plates for absence or presence of growth; any growth is considered significant. Read plates at 24 hours and record results. If there is no growth on the streptomycin plate, reincubate plate for an additional 24 hours.

Quality Control

Control strains are tested daily.

	Expected results **C**	**G**	**S**
E. faecalis (ATCC 19966)	+	+	-
E. gallinarum (ATCC 35038)	+	-	-
E. casseliflavus (ATCC 12755)	+	-	+

C = Growth Control; G = Gentamicin; S = Streptomycin; + = growth; - = no growth

Reporting Results

Blood cultures and sterile fluids --- Report with canned comment (Refer to Susceptibility Testing Manual).

Urines and other sites --- Do not report HLAR.

Reference

1. PML Technical Manual data sheet No. 323, Nov. 1993.

HIPPURATE TEST

Principle

This test determines the ability of bacteria to hydrolyse sodium hippurate. One of the end products, glycine is detected by the addition of ninhydrin reagent.

Reagents

Hippurate disks (store refrigerated)
Ninhydrin reagent
Sterile distilled water

Other Materials

Sterile tube (13 x 100mm)
Bacteriology loop
Sterile graduated pasteur pipette

Procedure

1. Place a Hippurate disk into a sterile tube and add 0.4 mL sterile water.

2. Heavily inoculate the tube with a loopful of the test organism.

3. Incubate at 35^0C for 2 hours.

4. Following incubation add 5 drops of ninhydrin reagent to the tube and shake gently.

5. Reincubate tube for 10 minutes and read reaction.

Interpretation

Positive: Deep purple-blue colour

Negative: No colour change or light purple

Precautions

1. A heavy inoculum is necessary to obtain a high concentration of enzyme.

2. Do not incubate longer than 30 minutes after addition of ninhydrin reagent because a false positive reaction could result.

Quality Control

Test with known positive and negative controls each time the test is preformed.

Positive: *Campylobacter jejuni* (ATCC 29428)

Negative: *Campylobacter coli* (CPI B7080)

Reference

1. Difco package insert.

INDOLE TEST

Principle

Bacteria that produce the enzyme tryptophanase will deaminate tryptophan to indole, pyruvic acid and ammonia in the presence of a co-enzyme pyridoxal phosphate.

Indole combines with Ehrlich's / Kovac's reagent to form a red-coloured complex.

Materials

Test A: Filter paper strips impregnated with Ehrlich's reagent.

Test B: Kovac's reagent
2% Tryptone broth (Difco, Oxoid)

Method

A: Filter paper strips are suspended over tubes of ONPG / PAM media, and incubated at 35^0C overnight.

Interpretation

Positive test - development of red colour on the strip.
Negative test - white-yellow colour.

B: 1. Inoculate the tryptone broth, and incubate at 35^0C overnight.
2. Add a few drops of Kovac's reagent to the broth.

Interpretation

Positive test - red colour in the upper layer.
Negative test - light-yellow colour in the upper layer.

Quality Control

Test the following positive and negative controls weekly:

Positive: *Proteus vulgaris* (ATCC 13315)
Negative: *Klebsiella pneumoniae* (ATCC 13883)

Reference

1. Murray, PA, et al. Manual of Clinical Microbiology 7th ed. 1999.

KOEHLER ILLUMINATION

The microscope should be set up using Koehler illumination for all parasitology examinations. This ensures that all the light from the lamp is being focused onto the specimen and that the field to be examined is evenly illuminated.

Procedure

1. Turn the lamp on.
2. Bring the condenser up to the top position, with the top lens swung in.
3. Open the condenser diaphragm.
4. Place a specimen on the stage and focus with the 10x objective.
5. Close the field diaphragm.
6. Lower the condenser until the edge of the field diaphragm is in sharp focus.
7. Center the field diaphragm image with condenser centering screws.
8. Open the field diaphragm until the edge just disappears from view.
9. Remove one eyepiece.
10. Looking down the eyepiece tube, close the condenser diaphragm until the illumination is approximately 2/3 full.
11. Replace the eyepiece.
12. Repeat for each objective lens when changed.

Reference

1. Baron E., Finegold S.M., Bailey & Scott's Diagnostic Microbiology, 8th ed., The C.V. Mosby Company, p64.

KOH STRING TEST

Principle

The formation of a string (DNA) in 3% KOH indicates that the isolate is a gram negative organism.

Reagents

3% KOH

Other Materials

Glass slides
Culture loop

Procedure

1. Place a drop of 3% KOH onto a glass slide.

2. Emulsify in KOH a loopful of the culture from a BA incubated for 18-24 hr.

3. Continue to mix the suspension for 60 sec and by slowly lifting the loop, observe for the formation of a string.

Interpretation

Positive: formation of a string within 60 seconds

Negative: failure to form a string

Precautions

1. False positive and false negative results may occur.

Quality Control

Known controls should be tested each time the test is performed.

Positive: *P. aeruginosa* (ATCC 27853)
Negative: *S. aureus* (ATCC 25923)

References

1. Murray PA et al. Manual of Clinical Microbiology, 7th ed., 1999; p. 1671.

LAP TEST

Principle

LAP (Leucine-β-naphthylamide) impregnated disks serve as a substrate for the detection of Leucine aminopeptidase. Following the hydrolysis of the substrate by the enzyme the resulting β-naphthylamine produces a red colour upon the addition of cinnamaldehyde reagent. This test is usually used, in conjunction with other tests, for the identification of streptococci and other catalase negative gram positive cocci.

Reagents

LAP discs
Cinnamaldehyde reagent (0.01% p-dimethylamino-cinnamaldehyde)
(disks and reagents are both in LAP kit)
Glass slide
Innoculating loop
Forceps
Sterile distilled water

Procedure

1. Place a LAP disk onto a glass slide and moisten it with one drop of sterile distilled water.
2. Rub a loopful of the culture onto the moistened disk holding it in place with sterile forceps.
3. Leave at room temperature for 5 minutes.
4. After 5 minutes, add 1 drop of cinnamaldehyde reagent.

Interpretation

Positive: red colour within one minute

Negative: no colour change or slight yellow colour

Quality Control

Test known positive and negative controls each time an unknown is run.

Positive: *E. faecalis* (ATCC 29212)
Negative: *Leuconostoc* (ATCC 8923)

Reference

1. Carr-Scarborough Microbiologicals package insert 1991.

MOTILITY TEST MEDIUM

Principle

Motility Test Medium is a semi-solid agar designed to demonstrate motility by diffusion.

Motility Test Medium is a modification of the formula of Tittsler and Sandhoizer. The medium contains small amounts of agar and gelatin, as well as triphenyltetrazolium chloride (TTC). TTC is a soluble compound which is taken up by the bacterial cells. Once the substance has been absorbed by the bacteria, it is reduced, releasing the acid formazan, a highly pigmented red, insoluble compound.

Organisms are stabbed into the medium with an inoculating wire. If the organisms are motile, they will diffuse into the soft medium laterally from the line of inoculation, resulting in a diffuse, pink color throughout the medium. Nonmotile organisms grow along the line of inoculation only, producing a pinkish-red line with no diffusion.

Storage

Upon receipt store at 2-8^{0}C away from direct light. Media should not be used if there are signs of contamination, deterioration (shrinking or discoloration), or if the expiration date has passed.

Limitations

Motility tests often show a false-negative reaction. The organism may be weakly motile, or the flagella may be damaged due to heating, shaking, or other trauma. A hanging drop motility may be performed from an inoculated tryptone broth incubated for 2-4 hours to confirm motility results. Consult appropriate microbiological texts for procedure.

TTC may be inhibitory to some fastidious bacteria.

Most motility of bacteria should be interpreted at 35^{0}C: however, certain bacteria such as *Yersinia enterocolitica* demonstrate the best motility at 25^{0}C.

Organisms that require oxygen for growth, such as *Pseudomonas aeruginosa,* will produce a spreading film on the surface of the medium, and will not fan out from the inoculation line where oxygen is depleted.

Procedure

1. Tube Method

   Prior to inoculation, the medium should be brought to room temperature. Inoculate selected colonies of a pure 18 to 24 hour culture, or from a turbid broth culture 4-8 hours old. Using a straight needle, stab the center of the medium about 1/4" from the top. Incubate the tubes with the caps loose at 35^0C (see "Limitations") for 18-24 hours. Observe for motility.

2. Plate Method

   If using a multipoint inoculation system, make a pour plate form the 18 ml tube by gently melting the agar in a boiling water bath and dispensing the liquid medium into a sterile petri dish. Prepare the inoculum by touching the top of one or two well isolated colonies and inoculating into a broth. Stab the inoculum into the medium using the modified pins of a replicator or by using a straight needle. Incubate aerobically at 35^0C (see "Limitations") for 16-18 hours. Examine for the presence of a pink diffusion from the point of inoculation.

Interpretation

Positive: A diffuse pink color occurring throughout the medium.
Negative: A pinkish red line at the stab site with no diffusion.

Quality Control

Test the following positive and negative control organisms each time the test is performed:

Positive: *Escherichia coli* (ATCC 25922*)*
Negative: *Klebsiella pneumoniae* (ATCC 13883)

References

1. Finegold, S.M., and E. J. Baron, Bailey and Scott's Diagnostic Microbiology, 7th ed., C.V. Mosby, St. Louis, 1986. Koneman, E.W., et al., Color Atlas and Textbook of Diagnostic Microbiology, J.B. Lippincott, Philadelphia, 1979. Lennette, E.H., et al., Manual of Clinical Microbiology, 4th ed., American Society for Microbiology, Washington, D.C., 1985.

2. MacFaddin, J.F., Biochemical Tests for Identification of Medical Bacteria, Williams and Wilkins, Baltimore, 1980. Tittsler R.P., and L.A. Sandhoizer, J. Bacteriol., 31:575, 1936.

MUG TEST (PGUA)

Principle

If an organism produces the enzyme glucuronidase it will break down the substrate ortho-nitrophenyl-beta-glucuronide liberating the ortho-nitrophenyl producing a yellow colour. This test is used, in conjunction with others, for the identification of *E. coli*.

Reagents and Materials

PGUA tablets
13x100mm tubes
Tryptone water
Kovac's reagent

Procedure

1. Prepare a dense suspension of the test organism (lactose-fermenter only) in 0.25 mL of the tryptone water.
2. Add 1 PGUA tablet to the tube.
3. Incubate at 36^{o}C for 4 hours.
4. Examine the tube for development of a yellow colour.
5. Add 1 drop of Kovac's Indole reagent and observe for the development of a red colour.

Interpretation

MUG positive: Yellow colour
MUG negative: Colourless

Indole positive: Red colour after addition of Kovac's
Indole negative: Kovac's remains yellow

MUG	INDOLE	INTERPRETATION / ACTOIN
+	+	report as *E. coli*
-	+	set up VITEK Identification
+	-	set up VITEK Identification
-	-	set up VITEK Identification

Precautions

1. *E. coli* O157:H7 and non-motile strains which produce verotoxin are MUG test negative.

Quality Control

The following controls are tested weekly:

	MUG	INDOLE
E. coli (ATCC 25922)	+	+
P. mirabilis (ATCC 12453)	-	-

Reference

1. Prolab package insert

NEISSERIA IDENTIFICATION SUGARS

Principle

The test determines the ability of bacteria to produce acid products from carbohydrates. Used as a method to identify Neisseria species and other fastidious organisms.

Materials

Cystine Proteose Peptone Agar (CPPA) media: - glucose, maltose, lactose, sucrose, control (no sugar).
Inoculating loop.

Procedure

1. For each tube, scrape a full 3 mm loopful of growth from the surface of a 24 hour chocolate agar subculture plate.
2. Deposit this inoculum a few millimetres below the surface of the medium.
3. Incubate at 35^{o}C.
4. Examine tubes after 1, 4 and 24 hours incubation.

Interpretation

Positive: Yellow colour at top of tube
Negative: Red (alkaline) to orange (neutral) colour.

Organism	Glu	Mal	Lac	Suc	Cont
N. gonorrhoeae	+	-	-	-	-
N. meningitides	+	+	-	-	-
M. catarrhalis	-	-	-	-	-

Precautions

1. Inoculum must be heavy.
2. False positive results may occur if tubes are incubated in CO_2.
3. Tubes that appear bright yellow should be gram stained to check for contamination.

Quality Control

The following controls are run each time the test is performed:

N. gonorrhoeae (ATCC 43069)
N. meningitidis (ATCC 13090)
M. catarrhalis (ATCC 8176)

Reference

1. Murray PA, et al. Manual of Clinical Microbiology, 7th ed., 1999; pp. 592-598.

ONPG TEST

Principle

This test is used to demonstrate the presence or absence of the enzyme B-galactosidase using the substrate ortho-nitrophenyl-D-galactopyranoside in order to differentiate lactose-fermenting from non lactose-fermenting organisms and in the identification of *B. cepacia*.

Reagents

ONPG disks (Store refrigerated)
Sterile saline

Other materials

Sterile tube (13 x 100 mm)
Bacteriology loop
Sterile graduated Pasteur pipette

Procedure

1. Place an ONPG disk into a sterile tube and add 0.2 mL saline.
2. Heavily inoculate the tube with a loopful of the test isolate.
3. Incubate at 35^{o}C for up to 4 hours.

Interpretation

Positive: yellow colour within 4 hours

Negative: colourless at 4 hours

Precautions

1. A heavy inoculum is necessary to obtain a high concentration of enzyme.

Quality Control

Test with known positive and negative controls each time the test is performed.

Positive: *E. coli* (ATCC 25922)
Negative: *P. vulgaris* (ATCC 13315)

References

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD., 1980, p120-128.

ONPG-PHENYLALANINE-MOTILITY MEDIUM (ONPG-PAM)

Principle

This test is used to determine an organism's motility, its ability to ferment lactose and produce phenylalanine deaminase. The medium is primarily used as a screening procedure for the detection of enteric pathogens.

Reagents

ONPG-PAM tube
10% Ferric chloride

Other materials

Culture wire

Procedure

1. Inoculate the ONPG-PAM tube by stabbing the centre of it to the bottom of the tube.
2. Incubate the tube in O_2 at 35^{o}C X 18 hours.
3. Read the tube for ONPG, motility and indole.
4. Add 2 drops of 10% ferric chloride solution and read the phenylalanine result.

Interpretation

ONPG:	positive:	yellow
	negative:	no colour change
Motility:	positive:	diffuse growth from line of inoculum
	negative:	growth does not spread from line of inoculum
Phenylalanine (PPA):	positive:	green
	negative:	yellow/brown

Quality Control

Test with control organisms each time a new batch of meda is prepared.

	ONPG	Motility	PPA
K. pneumoniae (ATCC 13883)	+	-	-
P. vulgaris (ATCC 13315)	-	+	+

References

1. Murray, PA, et al. 1999. Manual of Clinical Microbiology, 7th ed., American Society for Microbiology, Washington, D.C.

OPTOCHIN SENSITIVITY TEST

Principle

This test is used to determine an organism's susceptibility to the chemical optochin (ethylhydrocupreine hydrochloride) for the presumptive identification of *S. pneumoniae*.

Reagents

Bacto Optochin Disks (5 μg disk) Store refrigerated
Mueller Hinton Sheep Blood Agar (MHSBA)

Other Materials

Culture loop
Forceps
Cotton swabs

Procedure

1. Inoculate the suspected alpha haemolytic colony onto a MHSBA to obtain confluent growth.

2. Using aseptic technique place an optochin disk onto the surface of the inoculated agar. Press down with forceps.

3. Incubate at 35^{o}C in CO_2 for 18-24 hours.

Interpretation

Susceptibile: Zone of inhibition of at least 14 mm
Resistant: Zone of inhibition less than 14 mm

Quality Control

Test with known susceptible and resistant control strains weekly:

Susceptible: *S. pneumoniae* (ATCC 6303)
Resistant: Viridans Strep. (LPTP 8610)

References

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD., 1980, p245-249.

2. Difco package insert, July 1983.

OXIDASE (API STRIP)

Principle

This test determines whether an isolate produces oxidase enzymes. This test is mainly used, in conjunction with other tests, for the identification of gram negative organisms and *Bacillus* species.

Reagents

API Oxidase Reagent

1. 0.2% Aqueous ascorbic acid: Reconstitute ascorbic acid with 25 ml sterile distilled water. This solution may be refrigerated for up to 28 days. The expiry date must be written on the bottle.

2. N,N,N,-Tetramethyl-p-phenylenediamine-dihydrochloride:
   Reconstitute with 5 ml of the 0.2% aqueous ascorbic acid. It is recommended that this be re-constituted 4-5 hours before use. This solution may be refrigerated for up to 7 days at 2 - 8^{o}C. The expiry date must be written on the bottle.

Other Materials

API filter paper
API oxidase tray
Wooden applicator stick

Procedure

1. Place a filter paper in the oxidase tray and moisten entire paper with oxidase reagent. Allow to air dry. May be used for up to 1 week.
2. Transfer a portion of the colony to the filter paper using a wooden applicator stick.
3. Observe for 30 seconds.

Interpretation

Positive: Development of a purple colour within 30 seconds
Negative: No colour change

Precautions

Nichrome wire may cause false positive reactions.

Quality Control

Test daily with known positive and negative controls.

Positive:	*P. aeruginosa*	(ATCC 27853)
Negative:	*K. pneumoniae*	(ATCC 13883)

References

1. API Oxidase package insert 3/80.

2. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, p249-260.

OXIDASE (SPOT TEST DROPPER)

Principle

This test determines whether an isolate produces oxidase enzymes and is used for the identification of Neisseria species isolated from primary plates.

Reagents

Spot Test dropper. Store at room temperature.

Procedure

1. Hold the dropper upright and squeeze gently to crush the glass ampule inside the dispenser.

2. Add 2 - 3 drops directly to the colonies to be tested and observe for 30 seconds.

Interpretation

Positive: Development of a purple colour within 30 seconds

Negative: No colour change

Note: Colonies which are positive must be subcultured immediately since prolonged exposure will result in death of the organisms.

Quality Control

Test daily with known positive and negative controls.

P. aeruginosa (ATCC 27853) : positive
E. coli (ATCC 25922) : negative

References

1. Difco Spot Text Oxidase reagent package insert 1985.

2. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, p249-260.

PASTOREX STAPH PLUS TEST

Principle

A rapid slide latex agglutination test for the detection of clumping factor, capsular polysaccharide and protein A produced by most strains of *S. aureus*.

Reagents and Materials

Pastorex test latex suspension (store refrigerated)
Disposable reaction cards
Plastic stick

Procedure

1. Confirm the identification of a suspect Staphylococcus by Gram stain and catalase test.
2. Allow the latex reagent to warm to room temperature before use.
3. Shake the reagent so that all of the particles are resuspended.
4. Dispense one drop of latex test reagent in one of the circles on the reaction card.
5. Dispense one drop of negative control reagent in another circle on the card.
6. Emulsify 1 to 3 colonies into the test latex with a loop for 10 seconds.
7. Repeat step 6 for the negative control reagent.
8. Gently rock the card for 30 seconds and look for clumping.
9. Discard the card into a discard container.

Interpretation

Positive test: Clumping within 20 seconds with the test latex particles only.

Negative test: No clumping in either latex.

Uninterpretable test: Clumping in the negative control.

Precautions

1. False positive results may occur after 40 seconds.
2. False positive agglutination can occur with organisms other than staphylococci.

Quality Control

Test known positive and negative controls daily.

Positive: *S. aureus* (ATCC 29213)
Negative: *S. epidermidis* (ATCC 12228)

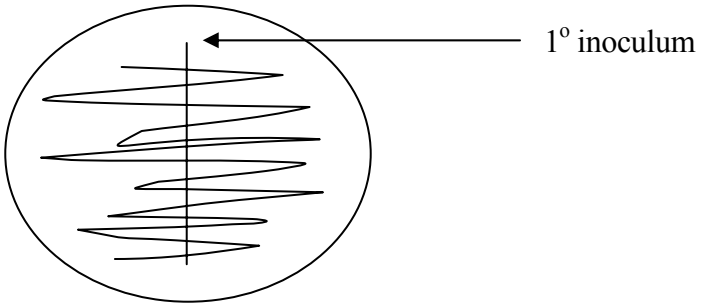
References

1. Pastorex Staph Plus package insert Feb. 1999.

PLATE STREAKING METHODS

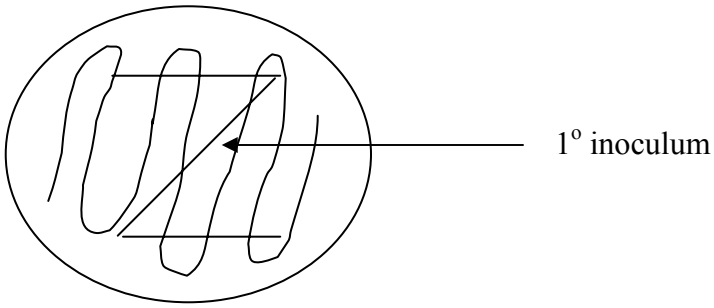
Blood Agar and MacConkey Agar for Urine Cultures

1 uL disposable loop
Inoculate in one continuous streak down the middle of the plate. With the same loop, streak out the entire plate at 90° to the initial inoculum. Streak a minimum of 15 lines.



Martin-Lewis Agar

Inoculate plate with specimen swab in a "Z" pattern across the plate (with continuous rotation of the swab while inoculating). Streak out the entire plate with a sterile loop at 90° to the initial inoculum. Streak a minimum of 15 lines.



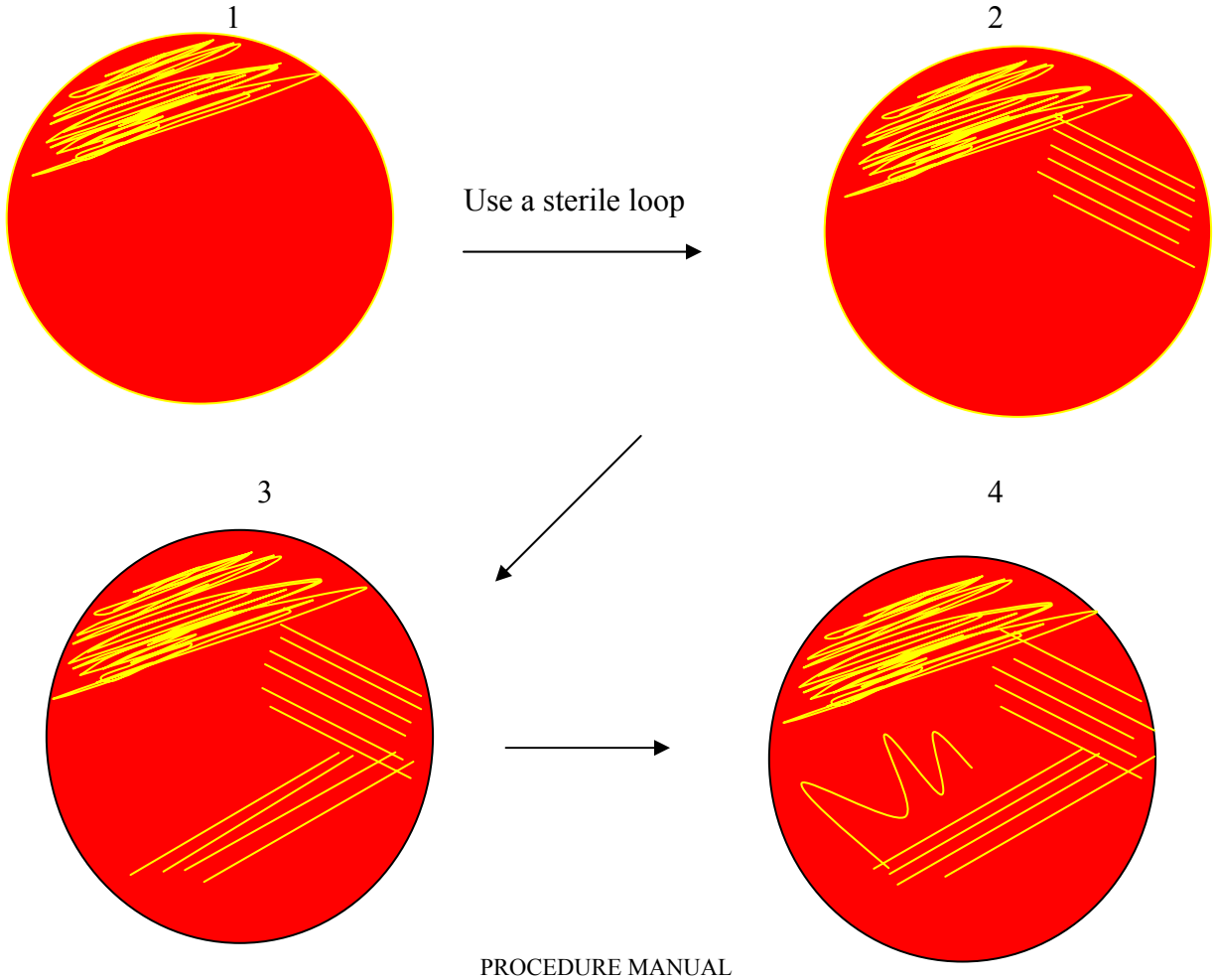
Isoplater Streaking

Growth Quantitation:

+ / -
1 +
2 +
3 +

Manual Streaking

Inoculate specimen with swab or loop onto the entire first quadrant of the agar plate. Use a sterile loop and streak out the second, third and fourth quadrants as per diagram:



PRO-AMP GLU-AMP TESTS

Principle

Rapid chromogenic tests for the identification of pathogenic Neisseria.

Reagents

Pro-Amp tablets
Glu-Amp tablets
Fast Blue BB solution
Sterile Saline

Other Materials

Sterile Tubes (13 x 100mm)

Procedure

1. Suspend the growth from Choc media in 2 tubes of 0.25 ml saline to achieve the turbidity > #2 McFarland standard.
2. Add 1 tablet to the respective tube.
3. Incubate at 36^{o}C x 4 hours.
4. After incubation add 3 drops of Fast Blue BB solution to each tube and read results after 10 minutes.

Interpretation

Positive: Orange/salmon colour
Negative: Yellow colour

Organism	Glu-Amp	Pro-Amp
N. gonorrhoeae	-	+
N. meningitidis	+	v

Quality Control

Test with control organisms when test is run:

N. gonorrhoeae (ATCC 43069)
N. menigitidis (ATCC 13090)

Reference

1. Pro lab package insert, February 1985.

PYR TEST

Principle

PYR (L-pyrrolidonyl-β-naphthylamide) impregnated disks serve as a substrate for the detection of pyrrolidonyl peptidase. Following the hydrolysis of the substrate by the enzyme the resulting β-naphthylamine produces a red colour upon the addition of cinnamaldehyde reagent. This test is used, in conjunction with others, for the identification of catalase negative, gram positive cocci including Enterococci and Group A Streptococci.

Reagents

PYR discs
Cinnamaldehyde reagent (0.01% p-dimethylamino-cinnamaldehyde)
(disks and reagents are both in PYR kit)
Glass slide
Innoculating loop
Forceps
Sterile distilled water

Procedure

1. Place a PYR disk onto a glass slide and moisten it with one drop of sterile distilled water.
2. Rub a loopful of the culture onto the moistened disk holding it in place with sterile forceps.
3. Leave at room temperature for 2 minutes.
4. After 2 minutes, add 1 drop of cinnamaldehyde reagent.

Interpretation

Positive: Pink or cherry red colour within one minute

Negative: No colour change or slight yellow colour

Quality Control

Test knows positive and negative controls each time an unknown is run.

Positive: Group A streptococcus (ATCC 19615)
Negative: Group B streptococcus (ATCC 13813)

Reference

1. Carr-Scarborough Microbiologicals package insert 1990.

QUANTITATION OF ORGANISMS & CELLS ON SMEARS & CULTURE

Microscopic:

	Report as:
± --- <1 per oil immersion field	±
+ --- 1 - 5 per oil immersion field	+
++ --- 5 - 10 per oil immersion field	++
+++ --- >10 per oil immersion field	+++

Culture:

	Report as:
± --- few colonies in primary inoculum	scant growth
+ --- confluent growth in primary inoculum	light growth
++ --- growth up to 2nd quadrant	moderate growth
+++ --- growth in or >3rd quadrant	heavy growth

Note: Quantitation precedes identification.

Size of colonies

lg - large
med - medium
sm - small
tiny - tiny
ppt - pinpoint

RapID ANA II SYSTEM

Principle

The RapID ANA II System is a qualitative micromethod employing conventional and chromogenic substrate for the identification of medically important anaerobic bacteria of human origin.

The tests used in it are based upon the microbial degradation of specific substrate detected by various indicator systems. The reactions are a combination of conventional tests and single-substrate chromogenic tests.

Materials

1. RapID ANA II panels
2. Suspension fluid
3. Kovacs spot indole reagent
4. RapID ANA II reagent
5. RapID ANA ID forms

Procedure

Make an equivalent McFarland #3 turbidity suspension of 18-24 hours AnO_2 culture (not more than 72 hours) in the supplied suspension fluid. Mix it thoroughly - can be used up to 15 minutes. Inoculate an agar (BA FAA) plate for purity and incubate for 24 hours anaerobically. Peel the lid off the panel marked "peel to inoculate". Using the Pasteur pipette, transfer the entire contents into the right upper corner of the panel. Seal the panel. Level the contents in the panel and slowly tilt the panel so that every chamber receives an equal amount of suspension. Incubate the panel at least four hours (not more than six hours) in non-CO_2 incubator at 35-37^{0}C. After the incubation period, read the panel prior to adding the reagents and write results on the ID form. Add the reagents as per instructions. Allow 30 seconds but not more than two minutes. Read it and score on the form.

Interpretation and Identification

Please follow the guidelines from the manufacturer and see the RapID ANA II ID Code Book.

See RapID ANA II System Insert #iii08-1/94 brochure which follows.

RapID MGP TEST

Principle

Rapid MGP Medium is a 5 hour test for the differentiation of *Enterococcus faecium* and *E. faecalis* from *Enterococcus gallinarum* and *E. casseliflavus* based on the ability to acidify the carbohydrate methyl-glucopyranoside (MGP).

Reagents

Rapid MGP Medium (Hardy Diagnostics)
Bacteriology loop

Procedure

1. Using a sweep of colonies from an 18-24 hour pure culture of the organism to be tested, stab the MGP media with the loop. There should be a visible cell paste on the loop as the media is inoculated.

2. Incubate aerobically at 35^0C for 5 hours.

3. Observe for the development of a yellow colour along the stab line indicating a positive test.

4. Reincubate weak reactions for 24 hours.

Interpretation

Positive: yellow colour along stab line
Negative: colour remains blue

E. casseliflavus	+
E. gallinarum	+
E. faecalis	-
E. faecium	-

Quality Control

Positive and negative controls are run each time the test is set up.

Positive: *E. casseliflavus* (ATCC 12755)

Negative: *E. faecalis* (ATCC 19966)

Reference

1. Hardy Diagnostics package insert 1999.

RapID VP TEST

Purpose

To aid in the identification of *S. milleri.*

Media

MR - VP broth

Procedure

1. Transfer approximately 0.2 ml of VP broth into a sterile 13 x 100 test tube.

2. Using a sterile inoculating wire inoculate the test organism heavily into the broth.

3. Incubate the tube at 35^0C for 5 hours.

4. After incubation add 1 drop of alpha-naphthol and 1 drop of 40% KOH.

5. Shake the tube gently for one minute to expose the medium to air. Allow 10-15 minutes for reaction to develop.

Interpretation

Positive - Red colour
Negative - No colour change within 10-15 minutes

Precautions

The order of adding reagents is important; alpha-naphthol followed by 40% KOH.

Reference

Ruoff, K.L., Ferraro, M.J. 1986. Presumptive identification of *S. milleri* in 5h J Clin Microbiol 24:495-497.

RapID YEAST PLUS TEST

Purpose

Used for the identification of yeast and yeast like organisms.

Materials

Rapid Yeast Plus Panel
Rapid Yeast Plus Reagent A and Reagent B
Rapid ID Inoculating fluid 2ml
Pasteur Pipettes
Cotton swabs

Procedure

1. Use a cotton swab suspend sufficient growth of the yeast in the Inoculating fluid to give a suspension heavy enough to obliterate the black lines on the inoculation card.

2. Peel back the panel lid over the inoculation port by pulling the tab marked "Peel to inoculate".

3. Using a Pasteur pipette transfer the entire contents of the inoculation fluid into the upper right hand corner of the panel and then reseal the panel.

4. Tilt the panel back away from the biochemical wells at approx. a 45% angle.

5. While tilting back gently rock the panel from side to side to evenly distribute the inoculum along the rear baffles.

6. Slowly tilt the panel forward toward the reaction cavities until the inoculum flows along the baffles into the biochemical wells.

7. Incubate panel at 30^0C for 4 hours.

8. After incubation peel the label lid from over the reaction cavities.

9. Add 1 drop of reagent A to cavities 7 to 14 inclusive.

10. Add 1 drop of reagent B to cavities 16-18 inclusive.

11. Read results after 30 seconds but no more than 1 minute after adding reagent. Record results onto supplied report form, and look results up in the Rapid ID Yeast Plus code book for interpretation.

Interpretation

Well #	**Positive**	**Negative**
1 to 5	Yellow	Blue to green
6	Yellow	Red, pink, orange, gold
7 to 14	Any yellow	Clear or cream
15	Red or dark red-orange	Yellow, yellow-orange or orange
16-18	Purple, red or dark pink	Clear straw, orange, pale to medium pink

Quality Control

Control strains are set up for each new lot number of panels.

References

1. Rapid ID yeast plus package insert issue #7/98.

SIM (SULFIDE-INDOLE-MOTILITY)

Principle

1. To determine the ability of an organism to liberate hydrogen sulfide (H_2S) from sulphur-bearing amino acids producing a visible, black colour reaction.
2. To determine the ability of an organism to split indole from the tryptophan molecule.
3. To determine if the organism is motile or non-motile.

This test is used, in conjunction with others, for the identification of *Enterobacteriaceae* when unable to identify using VITEK or API system.

Reagents

Kovac's Reagent

Other Materials

SIM Medium.
Inoculating wire or sterile glass pasteur pipette.

Procedure

1. With a pasteur pipette, draw up a small amount of previously inoculated TSB.
2. Stab vertically into the medium to within 1/4 to 1/2 inch from bottom: withdraw inoculating needle following line of inoculation.
3. Incubate O_2, 35^{o}C X 18-24 hours.
4. Add a few drops of Kovac's reagent and observe for development of a red colour.

Interpretation

H_2S production

(a) Positive: any blackening of the medium
(b) Negative: no blackening

Motility

(a) Positive: motile organisms migrate from the stab line and diffuse into the medium causing turbidity. They may exhibit fuzzy streaks of growth. (Compare with an uninoculated tube.)

(b) Negative: bacterial growth accentuated along stab line; surrounding medium remains clear.

Summary:

Results are recorded as follows. Remember that H_2S is first, then indole and finally motility.

-/-/- no H_2S, indole neg, non motile
-/-/+ no H_2S, indole neg, motile
-/+/- no H_2S, indole pos, non motile
-/+/+ no H_2S, indole pos, motile
+/-/- H_2S, indole neg, non motile
+/-/+ H_2S, indole neg, motile
+/+/- H_2S, indole pos, non motile
+/+/+ H_2S, indole pos, motile

Refer to Manual of Clinical Microbiology for specific organism reactions.

Precautions

1. An H_2S-producing organism may exhibit blackening on SIM medium, but none on TSI medium.
2. Some H_2S inhibition occurs when the temperature exceeds 34^{o}C.
3. Many bacteria are motile at one temperature and non-motile when at another.
4. If a motility test is difficult to interpret, compare with an uninoculated motility tube. If still in doubt, perform a wet prep or hanging drop preparation using a heavy loopful of an 18-24 hr culture.

Quality Control

Quality control must be performed on each new lot of SIM before being put into general use.

K. pneumoniae (ATCC 13883): -/-/-
P. vulgaris (ATCC 13315): +/+/+

References

1. MacFaddin JF, Biochemical Tests for identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, p162-173, 173-183, 214-218.

ACID FAST STAIN FOR MYCOBACTERIA (KINYOUN)

Principle

To stain Mycobacteria present in specimens and cultures.

Mycobacteria are different to stain with common aniline dyes. However, they will stain with basic fuschsin. Once stained, they retain the dye despite treatment with mineral acids i.e. HCl H_2SO_4. This property of acid fastness may be due to a lipd fraction called mycolic acid. Mycobacteria also exhibit degrees of resistance to decolourization with alcohol.

Materials

Kinyoun Carbol fuchsin
3% HCl in 95% ethanol
Brilliant green

Procedure

1. Prepare smear over an area of 2-3 sq. cm.

2. Heat fix smear on heating block (56^0C/1 hr). Then hold to incinerator for 10 secs.

3. Place slide on stain rack and allow to cool. Flood with Kinyoun Carbol fuschsin for 5 min.

4. Rinse off stain with water.

5. Decolourize with 3% acid alcohol for 3 mins.

6. Rinse with water.

7. Repeat decolourization for 1-2 mins. or until no red appears.

8. Rinse with water.

9. Flood slide 3-4 mins. with Brilliant green.

10. Rinse with water.

11. Air dry. DO NOT BLOT.

Microscopy

Place a drop of oil between the specimen and coverslip and oil again on top. Smears are examined with oil immersion lens. The coverslip prevents cross contamination. An average of 15 mins. is spent on each slide. The total area of the specimen must be examined.

References

1. Baker, F.J., Breach, M.R. 1980. Medical Microbiological Technique, p. 15

ACID FAST STAIN FOR NOCARDIA (MODIFED KINYOUN)

Principle

Nocardia species possess the unique characteristic of resisting decolorization with acid alcohol.

Reagents

1. Carbol-fuchsin
   - Basic fuchsin solution (3 g basic fuchsin in 100 mL 95% ethyl alcohol) 10 mL
   - Phenol 5% aqueous 90 mL

2. Decolourizer (1% sulfuric acid)
   - H_2SO_4 (concentrated) 1 mL
   - Distilled water 99 mL

3. Methylene blue
   - Methylene blue 0.3 g
   - Distilled water 100 mL

Staining Procedure

1. Fix the smear by gentle heating.
2. Flood the smear with Carbol fuchsin solution.
3. Allow the slide to stand for 5 minutes.
4. Wash the smear with tap water.
5. Decolorize the smear with 1% sulfuric acid until no more colour appears in the washing (approx. 1 min.).
6. Rinse with tap water.
7. Counterstain with methylene blue about 1 minute.
8. Rinse with tap water and air dry.

Interpretation

The filaments of *Nocardia* species and *Rhodococcus* appear red-stained against a blue background.

Quality Control

A positive control slide of *Nocardia* species is stained simultaneously with the clinical specimens.

References

1. Murray, PA. et al. Manual of Clinical Microbiology, 7th edition, 1999 ASM, Washington, D.C.

ACRIDINE ORANGE STAIN

Principle

Acridine orange is a fluorescent dye which will bind to the nucleic acid of bacteria and other cells. It is recommended for use for the detection of microorganisms in direct smears. It is useful for the rapid screening of specimens from normally sterile sites (eg. CSF) and blood smears, or smears containing proteinaceous material where differentiation of organisms from background material may be difficult.

Reagents

Acridine Orange spot test dropper. Stored at room temperature.
Absolute Methanol

Procedure

1. Prepare a smear of the specimen to be stained.
2. Allow to air dry.
3. Fix with methanol for 1 to 2 minutes.
4. Hold the dropper upright and squeeze gently to crush the glass ampoule inside the dispenser.
5. Flood the slide with the acridine orange and stain for 2 minutes.
6. Rinse thoroughly with tap water and allow to air dry.
7. Examine with a fluorescent microscope using low and oil immersion objectives.

Interpretation

Bacteria and fungus stain bright orange. The background appears black to yellow green. Leukocytes will stain yellow, orange and red.

Quality Control

Stain a smear of *Streptococcus pneumoniae* (ATCC 6303) each time the test is performed.

References

1. Difco Spot Test Acridine Orange Stain package insert, 1984.

BACTO 3-STEP GRAM STAIN PROCEDURE

Principle

To be used for problem smears to determine the Gram reaction of organisms.

Materials

3-Step Stabilized Iodine Technique

Bacto Gram Crystal Violet
Bacto Stabilized Gram Iodine
Bacto 3-Step Gram Safranin-S

3-Step Technical Iodine Technique

Bacto Gram Crystal Violet
Bacto Gram Iodine
Bacto 3-Step Gram Safranin-T

Microscope slides
Bunsen burner or methanol
Bacteriological loop
Swabs
Blotting paper
Microscope with oil immersion lens
Bactrol™ Gram Slide
Bactrol™ Disks

Procedure

1. Flood the fixed smear with primary stain (Bacto Gram Crystal Violet) and stain for 1 minute.
2. Remove the primary stain by gently washing with cold tap water.
3. Flood the slide with mordant (Bacto Stabilized Gram Iodine or Bacto Gram Iodine (traditional formulation) and retain on the slide for 1 minute. (Refer to LIMITATIONS OF THE PROCEDURE, #5)
4. Wash off the mordant with decolourizer / counterstain (Bacto 3-Step Gram Safranin-S or Bacto 3-Step Gram Safranin-T). (**NOTE**: Do not wash off iodine with water). Add more decolourizer / counterstain solution to the slide and stain 20-50 seconds.
5. Remove the decolourizer / counterstain solution by gently washing the slide with cold tap water.
6. Blot with blotting paper or paper towel or allow to air dry.
7. Examine the smear under an oil immersion lens.

Interpretation

REACTION	3-STEP TECHNIQUE using either Bacto Gram Safranin-S or Bacto Gram Safranin-T
Gram-positive	Purple-black to purple cells
Gram-negative	Red-pink to fuchsia cells

Quality Control

Run controls daily using 18-24 hour cultures of known gram-positive and gram-negative microorganisms.

EOSINOPHIL STAIN

Principle

A stain for the detection of eosinophils in clinical specimens.

Reagents

AJP Scientific Eosinophil stain:
- Solution I - Eosin Y
- Solution II - Buffer Ph 6.5
- Solution III - Methylene Blue
- Stored at room temperature

Procedure

1. Make a thin smear and spread evenly.
2. Fix slide by air drying or with gentle heat.
3. Cover slide with solution I and leave for 30 seconds.
4. Add solution II to cover slide. Mix gently and allow to stain for 3 to 5 minutes.
5. Wash off with tap water and drain.
6. Cover slide with solution III and immediately wash off with tap water. Drain and air dry.

Interpretation

Eosinophils stain with red cytoplasm and bright red granules.

Reference

1. A.J.P. Scientific INC package insert.

FUNGI-FLUOR™ STAIN

Principle

The Fungi-Fluor™ stain is used for the rapid identification of various fungal infections in fresh or frozen clinical specimens.

The active, fluorescing dye in the staining solution is Cellufluor which is the disodium salt of 4,4'-bis[4-anilino-6-bis-(2-hydroxyethel) amino-s-triazin-2-ylamino]-2,2'-stilbenedisulfonic acid. Fungi-Fluor™ staining solution is a 0.05% solution of this dye in deionized water with potassium hydroxide added as a clearing agent. The Fungi-Fluor™ counter staining solution B is an aqueous solution of Evans Blue dye used to reduce background fluorescence. Cellufluor binds nonspecifically to beta-linked polysaccharides found in the cell walls of various organisms such as chitin and cellulose.

Materials

Staining Solution A
Counterstaining Solution B
Absolute alcohol
Water
Fluorescent Microscope (250-400 nm filter)

Precautions

1. Store in a dark or opaque bottle, tightly sealed, at room temperature.

2. Avoid eye or skin contact: use gloves and protective glasses.

Procedure

1. Prepare smear of specimen and allow to air dry.

2. Fix on the rack with absolute alcohol for 5 mins. until dry. Fixed smears can be held indefinitely until ready to stain and examine.

3. Add few drops of Fungi-Fluor solution A (Cellufluor) for 1 minute.

4. Rinse gently with tapwater.

5. Apply coverslip to wetted slide and examine with the fluorescent microscope using the designated filter. If there is a delay, add distilled water to the coverslip just prior to examination. Use a fresh tube of water daily.

6. Optional for thicker smears. Add few drops of the counterstain Fungi-Fluor solution B. Rinse gently with tap water and then proceed as in step 5 above.

Quality Control

Stain a smear of *Candida albicans* daily.

Interpretation

Use 20x or 40x objective.

Fungal elements will appear yellow-green against a red-orange background when counterstain is used. Observe for characteristic morphology.

References

1. Manufacturers' Instructions (Data Sheet #316). Fungi-Fluor™ kit - Polysciences, Inc., July 1995

2. Clin. Micro. Newsletter 9:33-36, March 1, 1987.
K.L. McGowna. "Practical Approaches to Diagnosing Fungal Infections in Immunocompromised Patients".

3. J. Clin. Micro. 28:393-394, Feb. 1990. V.S. Baselski et al. "Rapid Detection of *Pneumocystis carinii* in Bronchoalveolar Lavage Samples by Using Cellofluor Staining".

GRAM STAIN

Principle

Bacteria can be recognized as gram positive (blue-black/purple) if they retain the primary dye complex of crystal violet and iodine in the face of attempted decolourization, or as gram negative (pink) if decolourization occurs as shown by the cell accepting the counterstain safranin.

Generally the mechanism of the Gram stain is: The fixed bacteria are stained with the triphenylmethane dye, crystal violet. Next the smear is flooded with Grams solution which oxidatively forms an insoluble complex with the crystal violet. The smear is then flooded with the organic solvent, acetone-alcohol. Depending on cell permeability the crystal violet-iodine complex will be washed from Gram negative bacteria in solvent but not from Gram positive bacteria. Upon counterstaining with safranin, organisms which had been discolorized by the ethanol (Gram negative) will stain pink. Gram positive organisms which retained the crystal violet will appear blue-black/purple microscopically.

Materials

Crystal violet solution
Grams Iodine solution
Acetone alcohol
Safranin solution

Procedure

1. Prepare the film on the slide and allow to air dry.
   DO NOT HEAT TO DRY FILM.

2. When film is dry, place slide on heating block for several minutes. Slide should be just warm to your hand.
   DO NOT OVERHEAT.

3. Allow slide to cool - this will happen quickly - in just a few seconds.
   DO NOT ADD STAIN TO HOT SLIDE.

4. Flood slide with crystal violet - leave 1 minute.

5. Wash gently with water.

6. Flood slide with Grams Iodine - leave 1 minute.

7. Wash iodine from slide with acetone-alcohol mixture. Add a few more drops of acetone-alcohol until no more colour comes from film - usually 30 seconds.

8. Wash gently with water.

9. Flood slide with safranin - leave 1 minute.

10. Wash gently with water. Clean back of slide with tissue and place slide in tray.

Precaution

1. At no time should the film (smear) be exposed to too much heat. When the specimen is still wet, heat causes coagulation of the protein resulting in heavy overstaining which cannot be removed by the decolourizer. A thick smear will also show more tendency to "lift off" during staining.

2. Rinsing the Grams Iodine off with the decolorizer gives more stability to the CV-GI complex and false over decolorizing will not take place.

3. Flooding a hot slide with crystal violet will cause the stain to precipitate and make decolourizing much more difficult.

Quality Control

It is recommended that controls be run concurrently with unknowns or at least run on a daily basis using known smears containing Gram positive and Gram negative bacteria.

STAPHAUREX TEST

Principle

A rapid slide latex test for the detection of clumping factor and protein A produced by most strains of *S. aureus*.

Reagents and Materials

Staphaureux latex suspension (store refrigerated)
Disposable reaction cards
Culture loop or wooden applicator stick

Procedure

1. Confirm the identification of a suspect *Staphylococcus* by Gram stain and catalase test.
2. Allow the latex reagent to warm to room temperature before use.
3. Shake the reagent so that all of the particles are resuspended.
4. Dispense one drop of latex reagent onto the reaction card.
5. Add 1-3 colonies to the drop, mix well with a loop or wooden applicator stick.
6. Rock the slide for 20 seconds and look for clumping.
7. Discard the slide into a discard container.

Interpretation

Positive test: Clumping within 20 seconds with the sensitized latex particles.
Negative test: No clumping

Precautions

1. False positive results may occur after 20 seconds.
2. False positive agglutination can occur with *E. coli* and *C. albicans*

Quality Control

Test known positive and negative controls daily:

Positive: *S. aureus* (ATCC 25923)
Negative: *S. epidermidis* (ATCC 12228)

References

1. Staphaurex Package insert, July 1992.

STREPTOCOCCAL GROUPING

Principle

This test is used to determine the Lancefield group of an isolate. Latex particles labelled with specific group antisera will agglutinate in the presence of the corresponding antigen after extraction with nitrous acid.

Reagents

Pro-lab Streptococcal grouping Latex kit.

Other Materials

Droppers
Disposable slides
Wooden stirring sticks
13x100 mm test tubes

Procedure

1. Label one test tube for each isolate.
2. Add one drop of Extraction Reagent 1 to each tube.
3. Suspend 4 beta-haemolytic colonies in the Extraction Reagent 1.
4. Add 1 drop of Extraction Reagent 2 to each tube.
5. Shake the tube and incubate for 2 minutes at RT.
6. Add 7 drops of Extraction Reagent 3 to each tube. Mix the reaction by vortexing the tube for 10 - 15 seconds.
7. Dispense one drop of each latex suspension to be tested onto separate circles on the test card.
8. Using a pasteur pipette, add one drop of extract to the latex suspension.
9. Mix the latex and extract with the wooden stick using the complete area of the circle.
10. Gently rock the card for 2 minutes and look for agglutination.

Interpretation

Positive: Strong visible agglutination within 2 minutes.

Negative: Milky appearance without visible agglutination.

Precautions

1. False positive reactions have been known to occur with organisms from unrelated genera eg. *E. coli*, *Klebsiella* sp., *Pseudomonas* sp.

Quality Control

Test reagents are checked weekly.

Each test should be tested with at least one extra grouping latex suspension as a negative control.

Reference

1. Pro-lab Streptococcal Grouping package insert.

TETRAZOLIUM REDUCTION TEST (TTC)

Principle

To differentiate between *E. faecalis* and *E. faecium*.

E. faecalis reduces the colorless compound (Tetrazolium-chloride) to an insoluble substance - formozan which is red.

Material

BHI broth/Tetrazolium-chloride (TTC)

Procedure

Inoculate a loopful of an overnight plate culture to 1 ml of TTC broth. Incubate at 35^0C and observe the reaction at 2 hours. If negative, reincubate up to 8 hours / overnight.

Interpretation

Positive - deep magenta
Negative - colourless or faint pink

Quality Control

Positive: *E. faecalis* (ATCC 29212)
Negative: *E. faecium* (ATCC 19434)

References

1. J. Gen. Microbiology (1965), 38, 279-287.

THERMONUCLEASE TEST

Principle

Staphylococcus aureus contains a heat-stable thermonuclease and coagulase negative staphylococcus does not. This is a rapid test to differentiate between the two organisms.

Materials

Toluidine blue-O DNA plate (Q-Lab)
13x100 mm tube with white cap
pasteur pipettes

Procedure

1. Dispense 2 - 3 mL of blood broth from BacT/Alert bottle showing gram positive cocci in clusters in the direct Gram stain into a sterile capped 13x100 mm tube.
2. Place tube in heating block, 100^0C for 15 minutes.
3. Let cool to room temperature.
4. Centrifuge at approximately 2500 rpm for 3 minutes.
5. Inoculate a pre-warmed (35^oC for 1 hour) toluidine blue-O DNA plate by filling wells (cut well with the end of a pasteur pipette) with 2 drops of the supernatant.
6. Incubate the plate at 35^oC in the upright position (agar side down).
7. Inspect the plate at, 1 hour, 2 hours and 4 hours and again after overnight incubation if negative at 4 hours.
8. Always run negative and positive control wells with each plate each day.

Interpretation

Positive: Pink zone of clearing at the edge of the well with a darker blue ring at the outer periphery of the zone; indicates thermonuclease activity

Negative: No zone or a small clear zone around the well

Quality Control

1. Inoculate 5 day negative patient BacT/Alert bottles with 0.5 mL of a slightly turbid suspension of (a) *S. aureus* (ATCC 25923) and (b) *S. epidermidis* (ATCC 12228) in trypticase soy broth.

2. Incubate the bottles overnight at 36^{o}C on the shaker.

3. Remove 3 - 6 mL of the broth-blood from the bottles and process in the same manner as the patient specimens (steps 1 to 4). Always QC new controls before use with patient specimen.

4. Supernatants may be kept refrigerated for up to 1 month for use as controls.

Reference

1. Rafner, H.B., & Stretton C.W. 1985. Thermonuclease test for same day identification of *S. aureus* in blood cultures. J. Clin. Microbiol. 21:995-996.

TRIBUTYRIN TEST

Principle

A rapid chromogenic test for the identification of *M. catarrhalis*.

Reagents

Prolab Tributyrin (TRIB) tablets.
Store at room temperature.
Sterile saline.

Other Materials

Sterile tubes (13 x 100 mm)

Procedures

1. Suspend the growth from CHOC in 0.25 mL (6 drops) saline to achieve the turbidity >#2 McFarland standard.
2. Add 1 tablet to the tube.
3. Incubate at 35^0C x 4 hours.
4. Examine the tube for development of a yellow colour.

Interpretation

Positive: Yellow/yellow orange colour

Negative: Red

Quality Control

Test the following organism weekly:

Positive: *M. catarrhalis* (ATCC 8176)
Negative: *N. gonorrhoeae* (ATCC 43069)

References

1. Prolab package insert, February 1985.

TSI (TRIPLE SUGAR IRON)

Principle

To determine the ability of an organism to attack a specific carbohydrate incorporated in a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulfide (H_2S) production. This test is used, in conjunction with others, for the identification of enteric pathogens.

Materials

TSI Slant
Inoculating wire or sterile glass pasteur pipette.

Procedure

1. Using the an inoculating wire, dip into the previously inoculated TSB.
2. Stab the butt of the TSI to within 1/4 inch from bottom, draw out and fishtail over slant. Do not tighten cap.
3. Incubate O_2, 35^{o}C X 18-24 hours.

Interpretation

Carbohydrate utilization:

1. Fermentation of glucose only
   (a) slant: red colour (alkaline reaction)
   (b) butt: yellow colour (acid reaction)

2. Fermentation of glucose and sucrose and/or lactose
   (a) slant: yellow colour (acid reaction)
   (b) butt: yellow colour (acid reaction)

3. Neither glucose nor lactose nor sucrose fermented
   (a) slant: red colour (alkaline reaction)
   (b) butt: (i) aerobic organism
   (a) No growth
   (b) No colour change
   (ii) facultative organism
   red colour (alkaline reaction)

Gas production:

1. Aerogenic:
   (a) Gas production: CO_2 and H_2
   (b) Evident by one of the following:
      (i) a single gas bubble
      (ii) bubbles in the medium
      (iii) splitting of medium
      (iv) complete displacement of the medium from bottom of the tube leaving a clear area
      (v) slight indentation of medium from the side of the tube

2. Anaerogenic:
   No gas production

H_2S production:

The presence of a black precipitate (ferrous sulfide) is evident by:
(i) A black colour spread throughout the entire butt masking the acidity; may even be a slight evidence on the slant
(ii) A black ring near the top of the butt area
(iii) A black precipitate scattered throughout the butt but not entirely masking the acidity present

Summary:

The ways of recording the TSI reactions are listed below. Remember that the slant is first, followed by the butt reaction.

acid/acid	+/+
acid/acid/gas	+/+ with gas
acid/acid/gas/H_2S	+/+ with H_2S
alkaline/acid	-/+
alkaline/acid/gas	-/+ with gas
alkaline/acid/gas/H_2S	-/+ with gas and H_2S
alkaline/acid/H_2S	-/+ with H_2S
alkaline/alkaline	-/-

Precautions

1. The TSI tube should be read within 18-24 hr. If read earlier, a false +/+ reaction may occur; if after 24 hr, a false -/-reaction may occur.

2. An H_2S organism may produce so much black precipitate that the acidity in the butt is completely masked. If H_2S is produced, an acid condition exists in the butt.

3. There is no inhibitor in this medium, therefore any organism may grow. Be certain that the organism tested is a catalase positive, gram negative bacillus.

4. *S. typhi* usually produces a ring of H_2S near the surface of the butt. Occasionally the amount of H_2S produced is so small that it will not be detected in TSI, but will show up in SIM media.

5. Some organisms produce such an abundance of gas that the medium may be completely displaced by gas, resulting in the medium being blown up into the cap of the tube. Use caution to avoid contamination.

6. Do not tighten the cap of a TSI tube. A free exchange of air is necessary to enhance the alkaline reaction of the slant.

Quality Control

Test the media each time it is prepared using the following organisms:

E. coli:	(ATCC 25922):	+/+
P. mirabilis:	(ATCC 12453):	-/+/H_2S
P. aeruginosa:	(ATCC 27853):	-/-

References

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, p183-194.

TUBE COAGULASE TEST

Principle

This test is used to speciate staphylococci by determining the ability of an isolate to clot plasma by producing the enzyme coagulase.

Reagents

Rabbit plasma

1. Reconstitute one vial at a time with sterile distilled water (volume determined by vial size).

2. Store refrigerated before and after reconstitution. Use within 72 hours of reconstitution.

Other Materials

Sterile glass tubes (tube method)
Culture loop or wooden applicator stick

Procedure

1. Add 0.5 mL of plasma to a sterile glass tube.

2. Emulsify a large loopful of a pure colony of *Staphylococcus* into the plasma.

3. Incubate at 35^{o}C for 4 hr, observing every 30 minutes for clot formation.

4. If there is no visible clot at the end of 4 hours, leave at room temperature overnight and observe for clot formation.

Interpretation

Positive: Clot formation

Negative: No clot formation

Precautions

1) When observing the tube, do not shake or agitate the tube.

Quality Control

Each time a coagulase test is performed, known positive and negative cultures must be tested.

Positive: *S. aureus* (ATCC 25923)
Negative: *S. epidermidis* (ATCC 12228)

References

1. MacFaddin, J.F., Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, pgs. 64-77.

UREA SLANT

Principle

To determine the ability of an organism to split urea by the action of the enzyme urease forming two molecules of ammonia with resulting alkalinity.

Materials

Urea Slant
Bacteriology loop

Procedure

1. From one isolated colony, heavily inoculate the urea slant.
2. Incubate O_2, 35^0C.
3. Read at 3 hours and again at 18-24 hours.

Interpretation

Positive: Intense pink-red colour.
- Rapidly positive: 1 to 6 hours (*Proteus* spp.)
- Delayed positive: ≥ 18 hours

Negative: No colour change

Precautions

Urea test media rely on the demonstration of alkalinity, thus are not specific for urease. The utilization of peptones or other proteins may cause an increase in pH.

Quality Control

Controls should be set up weekly.

P. mirabilis (ATCC 12453):	Positive - 4 hours
K. pneumoniae (ATCC 13883):	Weak positive - 18 hours
E. coli (ATCC 25922):	Negative

References

1. MacFaddin JF, Biochemical Tests for Identification of Medical Bacteria, 2nd ed., Williams and Wilkins, Baltimore MD, 1980, p298-308.

X AND V DISKS FOR IDENTIFICATION OF HAEMOPHILUS

Principle

Haemophilus spp. have different requirements for the growth factors X (hemin) and V (NAD). These requirements are determined based on the presence or absence of growth around disks impregnated with V,X and XV factors.

Reagents

1. Bacto Differentiation Disks
   - BV NAD and 125 units/ml bacitracin
   - BX hemin and 125 units/ml bacitracin
   - BVX NAD, hemin 125 units/ml bacitracin
   - Store refrigerated

2. Mueller Hinton Agar (MHA)

Other Materials

Forceps
Swabs
Inoculating loop

Procedure

1. Pick one colony from CHOC, taking care not to carry over any agar from the medium.
2. Inoculate MHA and streak over the entire surface of the plate using a sterile swab.
3. Place X, V and XV disks on the surface of the agar in the form of a triangle with at least 30-35 mm between disks.
4. Incubate CO_2, 35^{o}C X 18-24 hr.
5. Examine the pattern of growth around and/or between the disks.

Interpretation

Growth around the V and XV or the X and XV indicates a requirement for the single growth factor V or X respectively. Growth around only the XV disk indicates a requirement for both factors.

Precautions

1. Avoid carry-over of growth factors.

Quality Control

Known positive and negative controls must be set up in parallel with the test.

H. influenzae (ATCC 35056): Growth around the XV disk only
H. parainfluenzae (ATCC 7901): Growth around V and XV disks

References

1. Difco Package insert, June 1984.

XYLOSE FERMENTATION

Principle

A rapid chromogenic test for the identification of *E. gallinarum*.

Reagents

Prolab d-xylose tablets.
Sterile staline.

Other Materials

Sterile tubes (13 x 100 mm)

Procedures

1. Suspend the growth from BA in 0.25 mL saline to achieve the turbidity >#2 McFarland standard.
2. Add 1 tablet to the tube.
3. Incubate at 35 - 37^0C x 2 hours.
4. Examine the tube for development of a yellow colour.

Interpretation

Positive: Yellow / yellow orange colour
Negative: Red

Quality Control

The following organisms are tested weekly:

Positive: *E. gallinarum* (ATCC 35038)
Negative: *E. faecalis* (ATCC 29212)

References

1. J. Clin. Microbiol. 12, 620-623, 1980.