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Pure culture technique for medical post graduate students in microbiology

General considerations:

1. There are no uniform guidelines on performance of pure culture exercises. The methods vary across institutions and examiners.
2. Some examiners expect the identification to be performed using bare minimum reactions while others may expect the student to utilize all available test to demonstrate the positive and negative results of the organisms.
3. A disinfectant bowl, microscope, staining reagent, Bunsen burner, straight wire, bacteriological loops, glass slides, cover slips, marker pencil, control strains, magnifying lens, reagents and media must available before beginning the exercise.
4. The tables must be kept clean and tidy before the arrival of the examiners to the table. The results should be entered in the answer scripts, slides must be focused, tests kept ready and in order before the arrival of the examiners.
5. The student must always answer to the point and should not “beat around the bush”. Student should not argue with the examiner even if he/she is correct.
6. The student must be prepared with a logical and acceptable explanation for any of test performed/omitted or media chosen/ignored.
7. Positive and negative controls must be used whenever required.
8. The student should write down the media and tests required for the identification process (whether available or not).
9. Ideally the student should start taking growth for test from the given tube from bottom.
10. Gram stained smear, oxidase test, catalase test and hanging drop preparation are crucial in the identification. Many students go wrong here and everything after that obviously goes wrong.
11. Good streaking with several isolated colonies on agar plates is an advantage.
12. Despite best precautions, contamination of cultures can happen. Either the original tube might be (rarely) contaminated or may get contaminated during the process of identification resulting in isolation of more than one organism or confusing biochemical test results.
13. Inoculation of liquid medium must be preferably done using sterile Pasteur pipette. TSI agar, OF medium, LIA and citrate agar may be inoculated using straight wire.
14. Use of H_2S paper strips is more sensitive than TSI agar for H_2S detection.
15. The student must remember the commonly used ATCC or NCTC strains and reference centers of identification/typing of bacteria.
16. It is important to standardize the inoculum before performing disk diffusion test and zone of inhibition must be measured with scale before interpreting the results.
17. The student must be aware of the constituents of various culture media (indicators, pH etc) and their preparation.
18. The student must be aware of the principle, procedure, modification and interpretation of all the tests used in identification along with the suitable quality controls used for each of them.
19. The student must be aware of the various types of glasswares and their capacities.
20. The students must undertake sufficient care not to infect themselves, their neighbors or the examiners.

Usually pure cultures in the PG examination are given on nutrient agar slopes. If the organism is fastidious one, it is given on a blood or chocolate agar. When nutrient agar slope is provided, it almost rules out fastidious bacteria. When blood/chocolate agar slopes are provided one must think of streptococci, enterococci and Corynebacterium diphtheriae. Other fastidious isolates are rarely given. Although enterococcus and Corynebacterium diphtheriae can grow on nutrient agar, they are often given on blood/chocolate agar slopes but beware of this possibility.

DAY ONE:

Once the slope is given to you, examine the surface of the slope for colonial morphology. Observe if the growth is scanty, confluent or if isolated colonies are present. Presence of bluish-green pigmentation (and an odour) should give indication of Pseudomonas sps. Proteus sps may swarm all over the surface of the agar. Golden yellow pigmentation should indicate Staphylococcus aureus.

Colony morphology

Describe the colony morphology. Mention the approximate size (tiny, small, medium or large), margins, elevations, surface only if isolated colonies are seen. Mention the presence or absence of pigmentation. Sometimes the sentence ends with the following description, "…colonies seen extending from bottom to top". This is because when the slope is stroke inoculated, the inoculation begins from bottom and extends to top. Presence of hemolysis on blood agar may give some clues but the slope's thickness may obscure the hemolysis. Hemolysis, if present may be seen on the thin edges of the slope.

Next one must perform a gram stained smear. Colonies must be picked from the bottom of the slope and a smear must be prepared on one end of the glass slide. Remember to pick only small amount of growth. Three circles must be marked on the slide; one for the test smear and the remaining two for positive and negative controls. Use E. coli and S. aureus as gram negative and gram positive controls. Stain the slide and observe them under oil immersion objective. Gram stain is very crucial as this step decides the future steps. Most often students go wrong here. Sometimes, Klebsiella may appear like pneumococci. Enterococci may appear like bacilli. That is why the use of controls on the same slide is so important.

If gram negative bacilli are seen, all the cocci are excluded. The common possibilities include Enterobacteriaceae members, Pseudomonas, and Vibrio. The student should be aware that the examiner is at liberty to bring his/her own strains of bacteria and these bacteria may or may not be present in your own department. Presence of gram positive cocci could be Staphylococcus (clusters), Streptococcus (chains) or Enterococcus/Pneumococcus (pairs). Enterococci are usually arranged at angles (spectacle like) whereas pneumococci are parallel to the axis. Gram positive bacilli could be diptheroides or C. diphtheriae. Other bacilli are rare. Gram negative cocci are hardly ever given. These conditions vary from institution to institution.

If the isolate is gram negative bacilli, one must perform oxidase test and catalase test. Colonies from the bottom must be picked up using a sterile capillary tube and both the tests must be performed. Oxidase test is performed on dry oxidase strip/disc or moistened paper/disc. Pseudomonas and E. coli respectively are the positive and negative controls for the oxidase test. Many students go wrong in oxidase test. A positive oxidase should indicate Pseudomonas or Vibrio. The former could be more likely if pigmentation is also present. Catalase test is performed on slide or in test tube. For slide catalase test, colonies are picked up using sterile capillary tubes and transferred onto glass slide. Immediately one-two drops of 3% H_2O_2 (10 volumes) is placed over the colonies. For tube test, the capillary tube with colonies is dipped in a tube containing 3% H_2O_2. Immediate production of bubbles (effervescence) indicates positive test. If the slide test is performed the slide must be placed in a Petri dish that already contains a moistened blotting paper and then a lid must be placed over it. This is to prevent aerosol borne dissemination of the bacteria. The moist blotting paper is used to maintain the moisture till the examiner visits the table. A negative test rules out Shigella dysenteriae type 1; otherwise most commonly given gram negative bacilli are catalase positive. If gram positive cocci are seen, oxidase test has no relevance. One may ignore this test and directly proceed with catalase test,

which is performed as described above. While S. aureus is chosen for positive control, enterococci is the preferred negative control for catalase test. A negative catalase test indicates streptococci or enterococci.

If staphylococcus is suspected, one must proceed with slide coagulase test. One-two loopful of sterile saline is placed at either ends of a glass slide. One end is marked as control and the other as test. Colonies are picked up from sterile straight wire and emulsified on saline drops and a milky emulsion is prepared. One-two loopful of undiluted rabbit or human plasma is placed on the test suspension and mixed. Immediate appearance of clumps indicates positive test. The control saline emulsion should not show any clumps and if that occurs the positive test (even if seen) can't be relied upon. Thus the saline control is used to check autoagglutination. The student may proceed with other relevant tests such as tube coagulase test, mannitol fermentation (anaerobic), phosphatase test, DNAse test, tellurite reduction, salt tolerance (Mannitol salt agar) pigment production (Milk agar) etc depending the on the availability. Students are expected to write the required media and tests on the answer scripts. Although tube coagulase test is performed on 24-hour old broth culture, some centers prefer to setup the tube coagulase test immediately from the colonies. Three test tubes labeled test, negative control and positive control are filled with 1 ml of 1 in 10 diluted human or rabbit plasma. 0.5 ml of overnight broth culture or few colonies from the slope are transferred to the tube labeled test. Known S. aureus colonies are inoculated into tube labeled positive control and no organism is inoculated into tube labeled negative control. All the three tubes are incubated at 37^{o}C and observed every four hours for gelling/clotting of plasma. If the tubes remain negative until evening, the tubes must be taken out of the incubator and kept at room temperature until next morning. This is to prevent the lysis of the clot by fibrinolysin which may render the test negative if placed continuously in the incubator. Production of fibrinolysin doesn't occur at room temperature. Sugar fermentation tests are not performed for Staphylococci. However, the knowledge of the tests to differentiate Staphylococci from Micrococci may be useful here. The organism must be inoculated into two broths; one for further biochemical reactions and the other for antibiotic susceptibility testing and incubated. Some lesser important tests that may be performed include VP test and nitrate reduction besides anaerobic mannitol fermentation. A tube containing mannitol and indicator is placed in a water bath and boiled. After few minutes of boiling the surface of the broth is sealed with 1 cm layer of sterile liquid paraffin. After the tube cools down, a small inoculum of the organism in broth is delivered through the liquid paraffin using a sterile Pasteur pipette. The organism must be subcultured on blood agar and incubated. Some centers expect inoculation on nutrient agar too. While it is advised that the suspension for antibiotic sensitivity by disk diffusion should be prepared by directly emulsifying the colonies in saline, some centers expect a log phase growth in peptone water/nutrient broth. Only the antibiotics relevant for Staphylococci must be chosen. A Muller Hinton agar plate with 4% NaCl for 1 µg oxacillin disk for detection of MRSA may also be performed. Inclusion of 30 µg cefoxitin disk is reliable in detection of MRSA.

If gram positive cocci in chains are seen and the catalase test is negative, it strongly indicates streptococci. Presence of hemolysis and its type would indicate the possible identity of streptococci. The colonies may be emulsified in glucose broth or Todd-Hewitt broth (if available). Not many tests are available to distinguish the species in many centers. A blood agar plate may be inoculated to note the hemolysis; the streak lines may be stabbed if BHS is suspected. In order to differentiate S.pyogenes from S. agalactiae hippurate hydrolysis test, CAMP test, PYR test and susceptibility to 25 µg sulfamethoxazole and 0.04 units bacitracin disk may be performed. These tests depend on their availability at the centers. Serotyping (if available) may be performed to identify the streptococci. The knowledge of various ways of serotyping will be useful.

Gram positive cocci in pairs that are catalase negative could be either pneumococci or enterococci. Although cellular morphology may give a clue, some tests may be necessary to identify them. The organism should be inoculated on to blood agar for colony morphology and hemolysis. If enterococcus is suspected, it must be inoculated on to bile esculin agar and other tests such as PYR test, heat and salt tolerance tests may be performed to differentiate group D streptococci from enterococci. If

pneumococci is suspected tests such as bile solubility, inulin fermentation, quelling reaction may be performed. Antibiotics suitable to the most likely isolated must be selected. Vancomycin must be included if enterococci is suspected. Some examiners don't entertain any antibiotic susceptibility testing if beta hemolytic streptococci is identified.

Gram positive bacilli that are commonly given are C. diphtheriae and diphtheroids. If gram positive bacilli are seen in L/V arrangement, another smear must be prepared and stained with Albert's stain. Presence or absence of metachromatic granules must be noted for. Some institutions give C. diphtheriae on Loeffler's serum slop and the very appearance of Loeffler's serum slope sometimes gives away the identity. The organism should be inoculated on to blood agar and potassium tellurite agar and incubated at 37oC. Fermentation of glucose, galactose, maltose, sucrose, glycogen and starch in Hiss's serum water must be performed it identify diphtheria bacilli and subtype the three varieties. Nature of growth in meat broth can also help to differentiate among the varieties. If C. diphtheriae is suspected, toxigenicity test must be mentioned in the answer scripts. Some examiners expect the students to perform Elek's gel precipitation test (in dummy).

Gram negative bacilli throw up many challenges. Pigmented and oxidase positive bacteria are almost always Pseudomonas. If this organism is suspected, there are not many tests to perform. Motility may be observed by emulsifying small amount of colonies in saline or by hanging drop preparation. If the bacteria appear non-motile, a confirmatory hanging drop may be prepared after fours from a log phase growth in peptone water. The organism is subcultured on to nutrient agar (for pigmentation), MacConkey's agar (for lactose fermentation) and blood agar (for hemolysis). The organism may be inoculated into two tubes of nutrient broth of biochemical reactions and antibiotic susceptibility testing. The isolate must be inoculated into OF medium (oxidative or fermentative), nitrate test, TSI agar (inertness) and Simmon's citrate agar. Inoculum for citrate test must be from colonies and not from the broth suspension. Inoculation on to cetrimide agar may be used to demonstrate its resistance to cetrimide. Suitable antibiotics must be chosen keeping in mind the intrinsic resistances seen in Pseudomonas. If possible detection of metallo-beta-lactamases may be undertaken, given the availability in the center.

Gram negative, oxidase positive bacilli, which may or may not be curved and is non-pigmented indicates possibility of Vibrio. A hanging drop preparation must be setup to demonstrate active (darting-type) motility. An immobilization test can confirm the presence of Vibrio. If tentative motility turns out to be negative, a confirmatory motility may be performed four hours later. The organism is inoculated into two nutrient broth tubes and incubated. It is subcultured on to blood agar (hemodigestion), nutrient agar (distinct appearance & slide agglutination), MacConkey's agar (lactose fermentation), and TCBS agar (selectivity & sucrose fermentation). Biochemical tests include OF medium, TSI agar, IMViC tests, nitrate reduction, urease, gelatin liquefaction, sugar fermentation (sucrose, mannose, mannitol, arabinose) and utilization of amino acids (lysine, arginine and ornithine). Another peptone water tube may be inoculated for nitroso-indole test. Salt tolerance (0-10%) test and susceptibility to 150 µg O/129 and polymyxin B (50 units) may also be performed according to availability. Inoculation on CLED agar may be used to differentiate halophilic from non-halophilic vibrios. Although antibiotic susceptibility is not of prime importance, it may be performed using selected antibiotics.

For gram negative bacilli that are oxidase negative, there could be many possibilities among the Enterobacteriaceae family. Barring S. dysenteriae type 1, most other members are catalase test positive. A hanging drop preparation for detection of motility may detect Shigella and Klebsiella, which are non-motile. Non-motility in tentative hanging drop preparation must be confirmed by confirmatory hanging drop. Many students go wrong in interpreting hanging drop preparation. The organism must be inoculated into two tubes of nutrient broth for biochemical reactions and antibiotic susceptibility testing. The organism must be inoculated on to MacConkey's agar for lactose fermentation. While blood agar is not helpful in differentiating among the probable members of Enterobacteriaceae, some examiners insist on it. Nutrient agar may be inoculated for its use in slide

agglutination test. Biochemical tests include OF medium, TSI agar, IMViC tests, nitrate reduction, urease and sugar fermentation and utilization of amino acids decarboxylation (lysine, arginine and ornithine) and deamination (phenyl alanine). Suitable antibiotics may be chosen for antibiotic susceptibility testing. Knowledge of detection of ESBL and AmpC may be useful.

All the media and biochemical test must be incubated overnight at 37^{o}C except for the oxacillin disk for S. aureus where it must be incubated at 35^{o}C for 24 hours.

DAY TWO:

The student must begin the day two by observing the colonies on the inoculated agar plates for colony morphology. The colony morphology on each of the used medium must be documented and written in the answer script. The size, colour, surface, elevation, margin and pigmentation (if any), swarming (if any) must be observed and described. These plates must have well separated colonies and ones skill in streaking is put to test here.

The results of the biochemical tests must be written on the answer script. The order in which they are written varies from institution to institution and examiner to examiner. As far as possible, related tests must be clubbed together. The colony morphology and the biochemical reaction results should be used to identify the isolate. Some examiners expect a repeat gram stain, oxidase, catalase and hanging drop from the colonies whereas some don't. Unless contaminated, the culture plates must have colonies of single type only. Any additional tests to identify/confirm the isolate or subtype the isolate must be performed next. Differentiation of variants on tellurite containing agar may require incubation for 48 hours. Similarly VP test may require incubation of 48 hours and their results may not be available for interpretation.

If Vibrio cholerae is identified by biochemical reactions, it must be confirmed by slide agglutination using nondifferential O subgroup 1 antiserum. If positive, the isolated must be serotyped using ogawa and inaba antisera. Biotyping should be performed using chick RBC agglutination, VP test (if available), polymyxin B susceptibility and hemolysis. Vibrio can also be identified by performing string test, where a loopful of growth is mixed with a drop of 0.5% sodium deoxycholate in saline on a slide. Addition of few drops of concentrated H_2SO_4 to the 24-old peptone water culture gives cholera red reaction, which is a hallmark of Vibrio cholerae. Presence of surface pellicle in liquid medium is seen in both Pseudomonas and Vibrio. A positive urease test and phenyl alanine deaminase test identifies Proteae tribe. Presence of H_2S in TSI agar indicates common bacteria such as S. typhi, S. paratyphi B, S. typhimurium, Proteus mirabilis, Proteus vulgaris, or rarer bacteria such as Citrobacter freundi, Hafnia alvei and Edwardsiella tarda. Anaerogenic conditions (no gas during fermentation) is seen in S. typhi, Shigella and Vibrio. Pseudomonas is inert as far as fermentation reactions are concerned.

Identification strategy of common isolates (Escherichia, Klebsiella, Shigella, Salmonella & Proteus):
1) A gram negative bacilli that is oxidase negative, catalase positive, ferments glucose, and reduces nitrate is a member of Enterobacteriaceae family. Since it is oxidase negative both Pseudomonas and Vibrio are ruled out. A/A reaction in TSI agar and fermentation of lactose confirms that the isolate is lactose fermenter. Since it is lactose fermenter (in 18 hours), all non-lactose fermenters (such as Salmonella, Shigella, Proteus etc) are ruled out. This leaves with two options; E. coli and K. pneumoniae. Since the isolate is motile, produce dry colonies, indole positive, MR positive, citrate negative and urease negative; it is identified as E. coli.

2) A gram negative bacilli that is oxidase negative, catalase positive, ferments glucose, and reduces nitrate is a member of Enterobacteriaceae family. Since it is oxidase negative both Pseudomonas and Vibrio are ruled out. A/A reaction in TSI agar and fermentation of lactose confirms that the isolate is lactose fermenter. Since it is lactose fermenter (in 18 hours), all non-lactose fermenters (such as Salmonella, Shigella, Proteus etc) are ruled out. This leaves with two options; E. coli and K.

pneumoniae. Since the isolate is non-motile, produce mucoid colonies, indole negative, MR negative, VP positive (if available) citrate positive and urease positive; it is identified as Klebsiella pneumoniae.

3) A gram negative bacilli that is oxidase negative, catalase positive, ferments glucose, and reduces nitrate is a member of Enterobacteriaceae family. Since it is oxidase negative both Pseudomonas and Vibrio are ruled out. Since the colonies on MacConkey's agar non-lactose fermenting, both E. coli and K. pneumoniae are ruled out. K/A reaction in TSI agar and non-fermentation of lactose confirm that the isolate is non-lactose fermenter. Since the isolate is non-motile, all the motile NLFs (such as Salmonella & Proteus) are ruled out. Absence of H_2S production, negative citrate test, anaerogenic growth, negative urease test identifies the isolate as Shigella. Some stains of Shigella flexneri may produce indole. Shigella dysenteriae does not mannitol whereas other species do. Further serotyping can be done by slide agglutination test on glass slide using specific antiserum. Saline control to detect auto-agglutination must be performed simultaneously.

4) 3) A gram negative bacilli that is oxidase negative, catalase positive, ferments glucose, and reduces nitrate is a member of Enterobacteriaceae family. Since it is oxidase negative both Pseudomonas and Vibrio are ruled out. Since the colonies on MacConkey's agar non-lactose fermenting, both E. coli and K. pneumoniae are ruled out. K/A reaction in TSI agar and non-fermentation of lactose confirm that the isolate is non-lactose fermenter. Since the isolate is motile, Shigella is ruled out. The probable members are Salmonella and Proteus. Since the isolate is urease and phenyl alanine deaminase positive, it confirms the Proteae tribe and excludes Salmonella. Production of H_2S, positive citrate test, no fermentation of mannitol and negative lysine decarobylase test also adds to the identification of Proteus. Proteus mirabilis is indole negative, doesn't ferment maltose and ornithine decarboxylase positive whereas P. vulgaris is indole positive, ferments maltose and ornithine decarboxylase negative. Sucrose fermentation is unreliable in distinguishing these two species.

5) A gram negative bacilli that is oxidase negative, catalase positive, ferments glucose, and reduces nitrate is a member of Enterobacteriaceae family. Since it is oxidase negative both Pseudomonas and Vibrio are ruled out. Since the colonies on MacConkey's agar non-lactose fermenting, both E. coli and K. pneumoniae are ruled out. K/A reaction in TSI agar and non-fermentation of lactose confirm that the isolate is non-lactose fermenter. Since the isolate is motile, Shigella is ruled out. The probable members are Salmonella and Proteus. A negative urease and phenyl alanine deaminase test rules out the Proteae tribe. This leaves with only Salmonella. All salmonella are indole negative, sucrose fermentation negative, VP negative and MR positive. Salmonella typhi is anaerogenic, citrate negative and produces only small amounts of H_2S commonly seen at the interface of slant and butt. S. paratyphi A is citrate negative and H_2S negative whereas S. paratyphi B is positive for both H_2S and citrate test. Further identification of serotypes should be performed by slide agglutination using specific antiserum. A saline control must be undertaken during slide agglutination tests.

REPORT:

The exercise must end with a report describing the identity of the organism along with its antibiotic susceptibility testing report (if applicable).

- This article may not be in its complete form and may need some edition.
- Since the procedure and methods followed in this exercise widely varies, student is advised to follow the pattern according to the center's own policies.
- The contents of this article is based on my own experience & may be used under ones own risk.
- Document prepared on 18th March 2009.