Analyzing Unknown Bacterias Through Gram Staining, Selective, Differential, Fermentation Medias, IMVIC, Urease, Nitrate, Catalase and Oxidase

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ABSTRACT

Being given a broth numbered 14 with two unknown bacterias present, requires multiple tests that start with simple staining for arrangement and shape, to more in depth tests that can detect the enzymic activity and pH of the bacteria. Separating the two different bacteria was crucial with the help of selective agents in the Phenyl-ethyl Alcohol agar (PEA) and MacConkey agar(Mac). Thus further test were used like the IMVic multiple tests and more to describe the characteristics of the bacteria. Upon the final results, the gram positive bacteria was determined through process of elimination, to be *Staphylococcus epidermis* and the gram negative bacteria as *Escherichia coli.* For further tests that would examine unknown bacteria, to give better results, more than one of each test should be done. For instance instead of one PEA agar, two or three would suffice for a definitive answer. Also for the last set of test with the incubation of the IMVic etc, the incubation period shouldn't have been 4-5 days. Bacteria shows the best results when they are young, so an incubation period should be around 24-48 hrs. The number of tests should also increase to include gelatinase test, DNase test, temperature and pH testing to help identify more information on the unknown bacteria. This would help because some bacterias can show very similar results, therefore if increasing the different tests it could help narrow which bacteria is which.

INTRODUCTION

Microorganisms exist throughout our everyday lives whether within our bodies icing in digestion or a foreign obligated intercellular parasite causing diseases. A type of prokaryotic microorganism known as bacteria is always being exposed to us, despite not being able to see them without the help of a microscope. The importance of knowing what bacteria we are exposed to is ideal to help maintain a healthy life and for medical reasonings to diagnose a disease from a specific pathogenic bacteria which would help with choosing the right antibiotic to combat the bacteria quickly. This domain has about [1]5X40^30 of different bacteria on Earth that are ubiquitous so it is essential to categorize these bacteria into different phyla for easier organization and identification. Each bacteria exhibit several different characteristics whether they ferment sugar, are aerobic, produce acid, reduces nitrate, degrades urea using urease, breaks down citrate etc. Because each bacteria is so specific to their abilities, analyzing the unknown bacteria through a gram stain is essential at first[2], then a selective/differential, fermentation medias and the Indole, Methyl Red, Vogues-Proskauer and Citrate test (IMVic) with the multiple test systems concluding with the Urease, Nitrate, Catalase and Oxidase to help identify which bacterias are within broth number 14.

METHODS AND MATERIALS

[1]Whitman WB, Coleman DC, Wiebe WJ; Coleman; Wiebe (1998). “Prokaryotes: the unseen majority”. *Proceedings of the National Academy of Sciences of the United States of America* 95

[2] Gregersen, T. (1978) “Rapid Method for Distinction of Gram-negative from Gram-positive Bacteria” *European Journal of Applied Microbiology and Biotechnology* 123

One way to organize bacteria is through their outer membrane structure. Bacteria that have a large and thick peptidoglycan outer layer that take about 30-70% of the cell wall[3] are known as gram positive bacteria. A bacteria with a thinner small peptidoglycan layer and larger lipopolysaccharide and lipoprotein[4] layers are considered gram negative bacteria. Testing whether a bacteria is a gram positive or gram negative is done through a simple aseptic method known as gram staining. This technique allows gram positive bacteria to adhere to a crystal violet dye with the help of iodine that fixes the dye to the large insoluble complex of the gam positive. The staining also uses a safranin dye that would adhere to the gram negative after the gram positive is already dyed with the crystal violet. The gram staining technique also allows (with the use of a microscope) to see the shape and arrangement of the bacteria. Since the unknown broth #14 has both a gram positive and gram negative bacteria, after the staining, separating the different bacterias is essential to investigate exactly which gram positive and gram negative bacteria are present to use further test for bacteria characteristic. To separate the two, the use of selective agents like a Phenyl-ethyl Alcohol agar (PEA) and MacConkey agar(Mac) are used. The PEA agar was inoculated with the unknown broth 14 using a loop and aseptic techniques to grow for colonies. The PEA is specifically used because its selective agent is alcohol allowing gram positive bacteria to grow and suppressing and inhibiting gram negative bacteria's growth. This plate will have only my gram positive bacteria present. The MacConkey agar was also inoculated with the unknown broth 14 using a loop and aseptic techniques to grow for colonies. This selective

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[3]Vollmer W, Blanot D, de Pedro MA. 2008 Peptidoglycan structure and architecture. *FEMS Microbiol. Rev.* **32**, 149–167.

[4] Schleifer KH, Kandler O. 1972 Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Microbiol. Mol. Biol. Rev.* **36**, 407–477.

agent is bile salt and crystal violet, which will allow gram negative bacteria to grow and inhibit the gram positive bacteria. The Mac agar bacteria also has a differential agent as lactose/neutral red which will turn pink if the gram negative bacteria ferments lactose and produces lactic acid with a pH of 6.8 or less. This plate will only have my gram negative bacteria present. A third media type that uses a selective and differential agent is Mannital Salt agar(MS). This media was also inoculated with the unknown broth 14 using a loop and aseptic techniques to grow for colonies. The selective agent in MS is a high salt concentration of 15% only allowing the species of *Staphylococcus* to grow and turns yellow in the presence of *Staphylococcus aureus*[5]. This will tell me whether my gram positive bacteria is a *Staphylococcus* or not. *O*ne more media was used, an enrichment media of blood agar of 5% sheep blood. It is used to cultivate fastidious organisms like *Streptococcus*. Its differential in terms of hemolysis, either an incomplete alpha hemolysis, a complete beta hemolysis or a gamma with no hemolysis. Either gram positive or gram negative will show hemolysis. A control was also used on a agar plate. All five of these selective/differential medias were incubated at around 35 degrees Celsius for a period of 24-48hrs.

After incubation period, there were growths on all media. Results were written down and recorded. One recording that was obvious was growth on the MS agar without yellow change in color. it is now suggested and hypothesized that the gram positive bacteria must be *Staphylococcus epidermidis.* To take the investigation of finding out the gram negative bacteria and confirming the suspicions of the gram positive being *Staphylococcus epidermidis*, the IMVic tests are now performed. Fourteen separate test tubes are used for the IMVic tests and labeled as such; 2

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[5] Shittu, A., J. Lin, D. Morrison, and D. Kolawole. 2006. Identification and molecular characterization of mannitol salt positive, coagulase-negative staphylococci from nasal samples of medical personnel and students. J. Med. Microbiology

Triple Sugar Iron(TSI) slants, 2 urea broth(urea), 2 bile esculin slant(bile), 2 nitrate broth, 2 Sulfur Indole Motility(SIM), 2 Methyl Red Vogues-Proskauer(MRVP) and 2 Simmons Citrate(citrate). For one of each (1 TSI, 1 Urea, 1 bile, 1 nitrate, 1 SIM, 1 MRVP and 1 citrate) the gram positive grown on the PEA agar was inoculated into each, making sure to use careful aseptic techniques of heating the loop in between each inoculation. The tubes were labeled by their testing and also labeled PEA to distinguish that this is solely the gram positive bacteria. The TSI is used with a needle and the bacteria is stabbed into the agar once through and streaked on the slant. The TSI will tell whether the gram positive bacteria, whom which at this point is suspected to be *Staphylococcus epidermidis*, will ferment the 0.1% glucose first than the 1% sucrose and lactose in the media and whether it will produce gas. It is hypothesized that the *Staphylococcus epidermis* will ferment glucose and create gas, based on previous lab experiments done using *Staphylococcus* and the TSI slant. The bile and SIM are also stabbed using a needle with the gram positive bacteria and then streaked on top. Nitrate, citrate, urea and MRVP are inoculated with the loop using aseptic techniques. The same procedures are done for the remainder 7 tubes(1 TSI, 1 Urea, 1 bile, 1 nitrate, 1 SIM, 1 MRVP and 1 citrate) except using the gram negative bacteria that grew on the Mac agar. Once all 14 IMVic tests are inoculated with the bacterias, they will be incubated at around 35 degrees Celsius for a period of about 96 hours or 4-5 days.

After incubation period, the tubes are removed and growth is recorded. The first tube used was the SIM. If there is blackening of the media, there is sulfur reduction. The sodium thiosulfate in the medium could turn black if H_2S is present and reacts with the FeS. There is casein peptone in the SIM which is rich in tryptophan. Tryptophan converted into indole, pyretic acid

and ammonia using tryptophanase, a tryptophan enzyme. Ten drops of Kovacs Reagent are added to each SIM tube. If the broth turns red color, the bacteria is positive for indole, the bacteria's ability to produce tryptophanase enzyme. Next the MRVP tube was agitated, then split in half to two separate test tubes, one to test the Methyl red and one for the Voges-Proskauer, each tube labeled respectively. In the MR tube, 5 drops of methyl red are added to show a color change. If the bacteria turns red, it converted acetone acid and butanedoil to acid that has a pH of 4 or less. The tube for the VP shows the bacteria's ability to degrade glucose to basic or neutral end products, mainly acetylmethylcarbinol. Barrits reagent is added to the VP tube, which consists of potassium hydroxide and alcoholic alpha napthol. After adding the reagent, wait 15minutes for a reaction to occur. If the broth turns pink, it is a positive VP base acetoin reaction. If it turns brown it is a negative reaction creating an acidic reaction. (Note that a bacteria can never be both positive for MR, an acidic reaction and positive for VP, an alkaline reaction, but can be negative for both.) The next set of tubes of tubes used were the Simmons Citrate slant. The citrate test bacteria's ability to use citrate as its carbon source. The indicator is bromthymol blue which will change in color from green to blue if an alkaline reaction occurs. This alkaline reaction is the reaction of citrate releasing carbon dioxide that reacts with sodium and water to produce alkaline sodium carbonate. This will cause the bromothymol blue indicator to turn blue, giving a positive citrate reaction. The next test examined were the urease tubes. this test whether the bacteria can degrade urea to CO_2 + H_2O +NH_3 with the enzyme urease. The Urea broth is composed of peptone glucose urea buffer and phenol red, as the indicator that turns pink/red when the alkaline reaction has occurred. If the broth stays a yellow color, the pH is less than 6.8, an acidic reaction, a negative urease result. The next tubes examined were the nitrate tubes to test for the reduction

of nitrates to nitrites and ammonia with the enzyme nitrate reductase. 5 drops of sulfanilic acid reagent and 5 drops of dimethyl alpha napthylamine reagent are added to the tubes respectively. The nitrate broth is composed of 0.1% of potassium nitrate and 0.1% of agar, which reduces oxygen. The reduction of the nitrate is an anaerobic reaction, where the bacteria receives its oxygen from the nitrate. When the bacteria reduces nitrate to nitrite, it will react with the reagents a produce a red color, a positive nitrite reaction. If there is no color change shows that nitrates were not reduced or that the nitrites were reduced further. After the addition of the reagents and seeing if the react is positive or not, a pinch of Zinc powder is added to the broth of a negative reaction(no red color) to test if the nitrate were reduced further. If red color is shown, the nitrates were not reduced to nitrites. If there is no color change, the nitrates were reduced to ammonia or nitrogen gas. Next test was the catalase. Half of the PEA plate was used for the examination on the gram positive bacteria and half of the Mac plate was used on the gram negative bacteria. This test the bacterias ability to breakdown $2H_2O_2$ to $2H_2O + O_2$ with the enzyme catalase. The reagent hydrogen peroxide(H_2O_2) is added carefully and frugally to the half the plate, and if bubbling occurs, it is a positive catalase reaction when toxic H_2O_2 and superoxide were reduced during aerobic respiration in the electron transport chain. This will generally mean that bacteria that use oxygen and can tolerate it are catalase positive. The last test will use the other half of the plates that were used for the catalase test. This last test is the oxidase test that test for cytochrome C oxidase activity. Just like the catalase test, the oxidase test interacts with the electron transport chain, where cytochrome c is the enzyme that transfers electrons to oxygen in the final electron acceptor. To test this, two drops of the reagent tetramethyl-p-phenylenediamine are added to the untouched half of the PEA and Mac agars. The reagent is oxidized and gives up an electron, and

creates a maroon or purple blue color within the first 30 seconds. The color change indicates a positive oxidase reaction, with cytochrome c oxidize activity.

Once all tests are done and results are recorded, the tubes and plate are all discarded in a special bin that the instructor will discard in a sanitary and safe manner.

RESULTS

Table A IMVic, urease, nitrate catalase and oxidase results

IMVic,Ur, Ni, Ca, Ox Results

		TESTS						
		SIM	MR/VP	CITRATE	UREASE	CATA-LASE	NITRATE	OXI-DASE
BACTE-RIA	GRAM +	positive indole/ no black	positive MR/ negative VP	inconclu-sive	positive urease	positive catalase	complete reduction	negative oxidase
	GRAM -	positive indole/no black	positive MR/ negative VP	negative citrate	negative urease	positive catalase	positive nitrate	negative oxidase

Table A shows the results of the IMVic, urease, nitrate, catalase and oxidase results. The bile results were excluded due to cross contamination with a fellow peer accidentally using my bile slant. The citrate result for the gram positive bacteria were inconclusive because the citrate color expressed both positive and negative results.

Figure 1

In figure 1, the results for the gram positive bacteria shows that the TSI slant has a yellow butt and yellow slant, meaning the bacteria fermented glucose sucrose lactose and produced gas by the movement of the slant up- wards. there is no blackening of the media so the bacteria doesn't reduce sulfur and form H2S

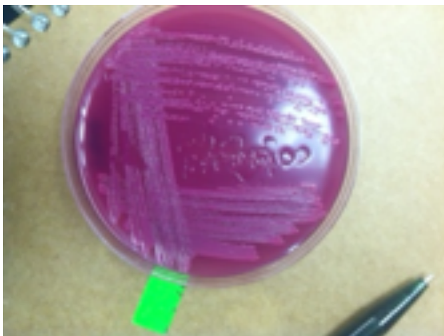

Figure 2

In figure 2, the results in tube labeled “TSI Mac” for the gram nega- tive bacteria show fermentation of glucose sucrose and lactose with gas production in the TSI slant.

Figure 3

In figure 3, there are many colonies for the gram positive bacteria that grew on the PEA agar, but not much else can be predicted from this alone.

Figure 4

Figure 4 shows the shiny, numerous and raised colonies of the gram negative bacteria on the Mac agar these colonies created a pink color indicating that the bacteria produced lactic acid.

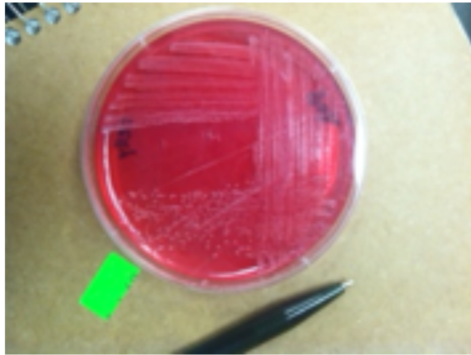

Figure 5

Figure 5 shows the results of the MS agar. there is positive *Staphylococcus* growth with out a golden yellow color. The assumption is that the bacteria must be *Staphylcoccus epidermidis.*

Figure 6

Figure 6 shows the growth of the negative and positive bacteria on the blood agar. It seems to either be an alpha incomplete hemolysis or gamma no hemolysis. Interpretation of the results aren't definitive

The gram staining gave an image of both the gram positive bacteria and gram negative bacteria. Because the gram stain worked, the bacterias aren't *Mycobacteria* or *Nocardia,* which require acid fast staining. The gram positive bacteria was dyed crystal violet on its large peptidoglycan layer and showed circular or coccus clusters. This rules out any *Bacillus subtilis* bacteria. The gram negative bacteria gave a safranin color from the counter stain and showed a rod like shape with an inconclusive arrangement. The PEA agar showed numerous clones for the gram positive bacteria as did the Mac for the gram negative. The Mac also showed a pinkish color of the bacteria indicating that the bacteria ferments lactose. This rules out *Serratia marcescens* as the gram negative because it does not ferment lactose. The MS agar had positive growth without any yellow coloring, allowing for the prediction of a *Staphylcoccus,* mainly *Staphylcoccus epidermidis,* ruling out *Staphylococcus aureus, Enterococcus faecalis.* The blood agar was examined and seemed to be either an alpha hemolysis or gamma hemolysis. It was definitely not a beta complete hemolysis so *Streplococcus* was ruled out as the gram positive because *Strepococcus* produces a positive complete beta hemolysis on erythrocytes. The TSI slants both showed fermentation of glucose, sucrose/lactose with gas production from the yellow slant and yellow butts. There were no blackening of the medias, therefore sulfur was not reduced. This excludes *Pseudomonas aeruginosa* which produces no fermentation and *Proteus vulgarus* and *Enterobacter* aerogenes which reduce sulfur. The SIM test showed a red color so they were positive for indole for both bacterias, no sulfur reduction again and slight motility. This rules out *Micrococcus luteus* as the gram positive bacteria because it does not have motility. The MR test were positive for both and negative for the VP test. This indicated that both bacteria showed an acidic pH over an alkaline one. With the citrate test, the gram negative bacteria had no reaction and the slant

stayed a green color. The gram positive bacteria had inconclusive results because the slant showed a green and blue color throughout. The slant must have had cross contamination, therefore results will be discarded. The urease test was positive for the gram positive bacteria so it can degrade urea while the gram negative doesn't degrade urea, giving a negative result. The nitrate test showed a positive result for the gram negative bacteria, turning red in the initial addition of the two reagents. The gram positive bacteria was colorless with the two reagents, therefore zinc was added to it. There was no color change, resulting in complete reduction of nitrate(NO3) into ammonia (N2). The catalase test was positive for both the gram negative and gram positive bacteria, showing that the bacterias are either aerobic, facultative anaerobes or microaerophiles that use the aerobic respiratory pathway during the breakdown of carbon for energy. *Clostridium* is ruled out, leaving just the predicted *Staphylococcus epidermis* as the gram positive and narrowing the gram negative to *Escherichia Coli*. To confirm the results, the oxidase test showed both negative results for both which is suspected for *Staphylococcus epidermis* and *Escherichia Coli.*

DISCUSSION

Based on the gathered information and recorded results, the gram positive bacteria is *Staphylococcus epidermidis*, while the gram negative bacteria is *Escherichia coli.* The selective and differential agar media were able to tell the gram positive bacteria right away from the positive growth on MS agar without a yellow change in color. The IMVic tests further confirmed hypothesis allowing for a positive MR result, a negative VP, a positive SIM result, positive glucose, sucrose and lactose fermentation with gas produced, positive urease, negative oxidase, positive catalase test. With the Selective/ differential media, not much was identified for the gram negative bacteria except that it grew on the Mac agar with a lactose fermentation, producing lactic

acid which was the pink color of the colonies. The IMVic test were able to distinguish more characteristics of the gram negative bacteria. The gram negative bacteria had positive glucose lactose and sucrose fermentation with gas produced and no blackening[6], was positive for indole or the SIM test, positive for MR and negative for VP, negative for citrate, negative for oxidase, positive for nitrate and negative for urease. Comparing these results with the use of process of elimination, the gram negative bacteria is *Escherichia coli*.[7] It didn't produce H2S like *Proteus*, fermented lactose unlike *Serratia* and had a positive MR unlike *Enterobacter*.

For further tests that would examine unknown bacteria, to give better results, more than one of each test should be done. For instance instead of one PEA agar, two or three PEA agars should be given for a definitive answer, especially since some media can get contaminated during incubation or around other undergraduate student who can mistakenly use your media by accident. Unfortunately my bile slants were discarded because a fellow peer accidentally inoculated it with his bacteria as well. If more than one bile slants were used, some sort of information could have been obtained from those. Also for the last set of test with the incubation of the IMVic etc, the incubation period shouldn't have been 4-5 days. Bacteria shows the best results when they are young, otherwise older bacteria can exhibit characteristics of gram negative bacteria.[8] So an incubation period should be around 24-48 hrs. The number of tests should also increase to include gelatinase test, DNase test, temperature and pH testing to help identify more

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[6]E.E. Geldreich, H.F. Clark, P.W.Kabler, C.B.Huff, and R.H. Bordner (1958) “TheColiform Group II. Reactions in EC Medium at 45 C” *Robert A. Taft Sanitary Engineering Center, Public Health Services, Department of Health, EDucation, and Welfare, Cincinnati, Ohio*

[7]Perry C.A. and Hajna ,A.A. (1944) “Further evaluation of EC medium for the isolation of coliform bacteria and Escherichiacoli.” *Am. J. Public Health, 34,735-738*

[8] Microbio Lab, Bio 3302L:New York City College of Technology Biol. Sciences (2013) *USA The McGraw-Hill Companies, 351*

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