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Accounts of Biotechnology Research

Volume 2 Issue 2

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AOBR, 2(2) 2015 [065-082]

Guidelines for quick application of biochemical tests to identify unknown bacteria

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ABSTRACT

In many scientific studies, researchers have used many unknown bacteria. The time required for reporting and identifying these bacteria must be evaluated. Biochemical tests are a conventional and inexpensive means of identifying bacteria. That is why many researchers prefer to apply these techniques in identifying microorganisms, instead of opting for newer techniques like polymerase chain reaction (PCR). Although identification of bacteria via biochemical tests is a well-known procedure among microbiologists, it consists of many complex procedures, making it very difficult for experts in other fields of study, such as environmentalists, chemists, or even biotechnologists and biologists, to evaluate given the lack of a quickly available, short, and simple reference manual. The goal of this paper is to present a systematic procedure that is completely understandable and applicable for researchers who are not familiar with microbiology or its relevant techniques.

KEYWORDS

Microbial techniques;
Microorganism identification;
Biochemical tests;
Microbial manual.

INTRODUCTION

In many scientific studies, researchers use different types of unknown bacteria, and reporting the identification of bacteria related to their area of research is necessary[1-6]. At first glance, bacterial identification is a very complex procedure that must be carried out by a microbiologist. However, for other researchers working in different fields, such as civil engineering, environmental engineering, chemical engineering, etc., the easy identification of bacteria is very difficult until they possess sufficient knowledge about biochemical tests and reliable references in the field of microbiology. Although many high-tech methods have been developed recently for the identification of bacteria, such as polymerase chain reaction (PCR), biochemical tests are still widely applied for several reasons. These include tests that are inexpensive, reliable, and easily applied in all laboratories. The goal of this paper is to develop a standard and simple protocol for the identification of unknown bacteria with a focus on biochemical testing requirements.

As a first step in bacteria identification, two tests, including gram and morphological characteristic tests, should be carried out. Through these tests, bacteria are

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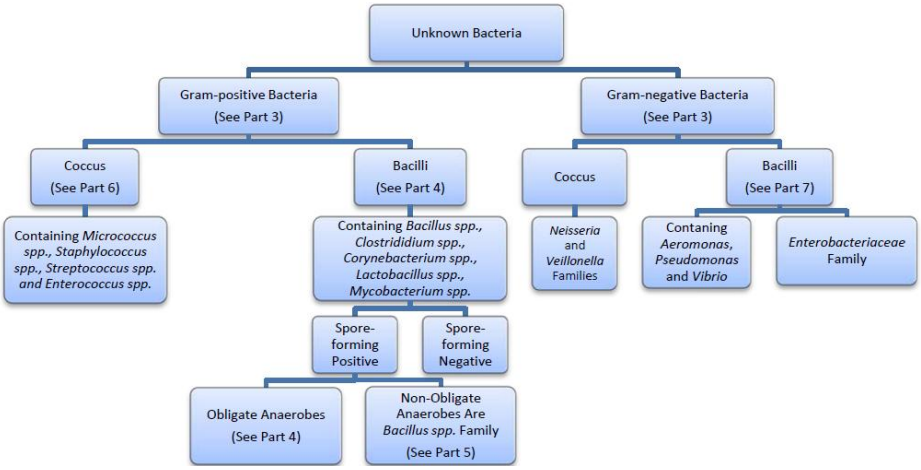

Figure 1: Identification chart of bacteria

categorized into smaller groups. Then, by using other tests, bacteria are divided into still smaller and smaller groups until they are identified (Figure 1).

CLASSIFICATIONS OF BACTERIA BY MORPHOLOGICAL CHARACTERISTICS

Generally, based on morphological characteristics, bacteria are divided into two major categories, those with round-ended cylinders (bacillus) and those with spheres (coccus). However, there may be others, such as helically twisted cylinders (spirochetes), cylinders curved in one plane (selenomonads), and unusual morphologies (such as the square *Archaea Haloquadratum*). Various shapes of bacteria are shown in Figure 2. Morphological characteristics of unknown bacteria can be directly examined by light microscope[11].

As displayed in Figure 2, coccus shapes are generally spherical or nearly spherical. Describing a bacterium as a coccus, or sphere, distinguishes it from a bacillus, or rod. The first step to identify bacteria is to examine them by light microscope to discern whether the bacteria are coccus or bacillus[12].

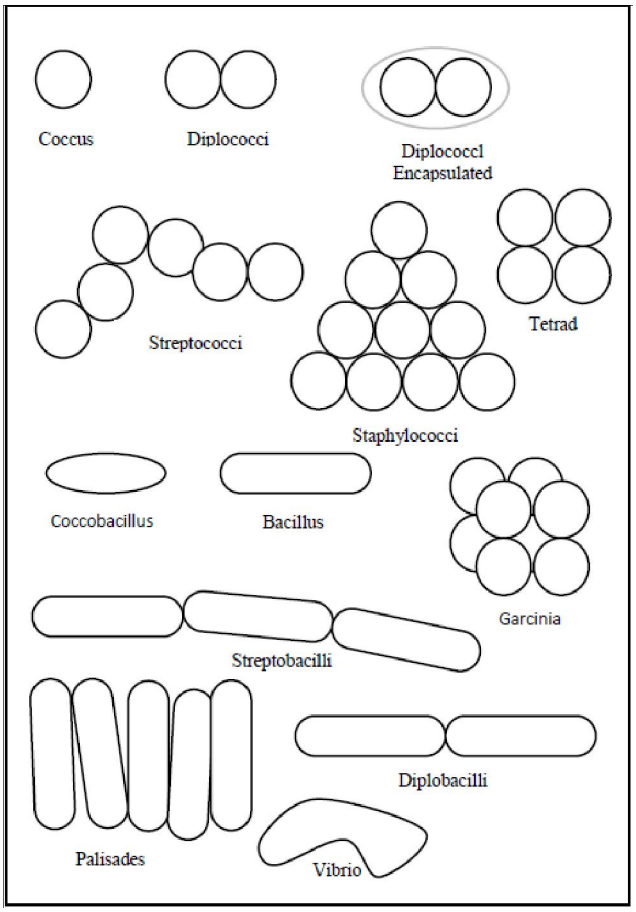

Figure 2: Different shapes of bacteria

CLASSIFICATIONS OF BACTERIA BY GRAM TEST

The Gram test is named after its inventor, Hans Christian Gram. The Gram test differentiates bacteria into two large categories, Gram-negative and Gram-positive. The Gram test can distinguish Gram-positive and Gram-negative bacteria based on the physical and chemical properties of their cell walls. This test is commonly among the first tests applied to identify bacteria. To apply the Gram test, first bacteria should be purified and then small amount of bacteria colonies is transferred by inoculation loop onto a slide. If bacteria are to be taken from a slant culture or Petri dish, a drop of

TABLE 1: Gram-positive bacteria based on morphological observation

Coccus	*Bacillus*
Streptococcus	*Corynebacterium*
Peptostreptococcus	*Listeria*
Enterococcus	*Erysipelothrix*
Staphylococcus	*Mycobacterium (Acid Fast)*
Micrococcus	*Propionobacterium*
Peptococcus	*Bacillus clostridium*
	Actinomyces
	Arachnia
	Nocardia (partially acid-fast)
	Streptomyces
	Erysipelothrix
	Lactobacillus
	Eubacterium

TABLE 3: Categorization of gram positive rod bacteria based on ability to form spores

Able to form spores	Unable to form spores
Bacillus spp.	*Corynebacterium spp.*
Clostridium spp.	*Lactobacillus spp.*
	Mycobacterium spp.

physiological saline (9 g NaCl in 1 l of distilled water) should be added to the slide[12]. Note that only a small amount of bacterial colonies is needed. After fixing bacteria on the slide by gentle flame and washing the fixed bacteria with crystal violet dye, iodine solution, decolorize and fuchsin solutions, respectively, results can be observed under light microscope. All Gram-negative bacteria appear as red or pink and Gram-positive bacteria are blue in color[13]. After Gram testing and observation of bacterial morphology, bacteria can be divided into four categories: 1) Gram-positive coccus, 2) Gram-positive bacillus, 3) Gram-negative coccus and 4) Gram-negative bacillus (TABLES 1, 2).

GRAM-POSITIVE RODS

Gram-positive rod bacteria include the *acillus spp.*, *Clostridium spp.*, *Corynebacterium spp.*, *Lactobacillus spp.*, and *Mycobacterium spp.* families. Following the biochemical test, further testing can be carried out in order to identify each one of the above-mentioned bacteria. Bacteria are tested to distinguish their ability to form spores. If they are positive for forming spores, they can be one among the following bacterial families: 1) *Bacillus spp.* or 2) *Clostridium spp*. If the bacteria

TABLE 2: Gram-negative bacteria based on morphological observation

Coccus	Bacillus
Neisseria	*Acinetobacter*
Veillonella	*Moraxella*
	Brucella
	Bordetella
	Bacteroides
	Cardiobacterium
	Fusobacterium nucleatum
	Haemophilus
	Bacteroides
	Francisella
	Pasteurella
	Actinobacillus
	Eikenella
	Spirillium
	Campylobacter
	Vibrio
	Fusobacterium
	Streptobacillus
	Flavobacterium

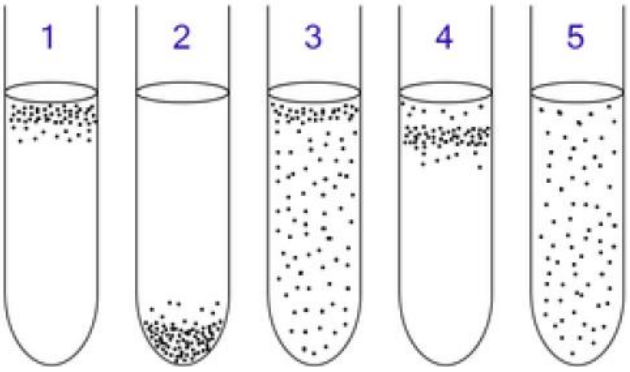

Figure 3: Growing forms of different bacteria to determine their oxygen requirements

are not able to produce spores, they belong to one of the following families: *Corynebacterium spp.*, *Lactobacillus spp.* or *Mycobacterium spp.* (TABLE 3)[14].

Able to form spores

In this step, bacteria are tested to distinguish whether they are obligate anaerobes or not. If they are obligate anaerobes, they are from the *Clostridium spp.* family. If they are not obligate anaerobes, they are from the *Bacillus spp.* family. If bacteria are members of *Bacillus spp.*, refer to Section 5 to see how one can identify all family members of *Bacillus spp.*

Aerobic and anaerobic bacteria can be identified

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by growing them in tubes of broth (see Figure 3). Thioglycollate can react with oxygen and consume it. Therefore, in a tube of Thioglycollate broth, oxygen concentration is decreased with increasing tube depth. In other words, the concentration of oxygen on the surface and bottom of Thioglycollate broth is highest and lowest, respectively. In this test, position of bacterial colonies determines whether they are aerobic or anaerobic (see Figure 3)[15].

1. Obligate aerobes need oxygen because they cannot ferment or respire anaerobically. They gather at the top of the tube, where oxygen concentration is higher.

2. Obligate anaerobes are poisoned by oxygen, so they gather at the bottom of the tube where the oxygen concentration is low.

3. Facultative anaerobes can grow with or without oxygen because they can metabolize energy aerobically or anaerobically. They gather mostly at the top because aerobic respiration generates more ATP (adenosine triphosphate) than either fermentation or anaerobic respiration.

4. Microaerophiles need oxygen because they cannot ferment or respire anaerobically. However, they are poisoned by a high concentration of oxygen. They gather in the upper part of the test tube but not at the very highest position.

5. Aerotolerant organisms do not require oxygen, as they metabolize energy anaerobically. Unlike obligate anaerobes, however, they are not poisoned by oxygen. They can be found evenly spread throughout the test tube.

Unable to form spores

When bacteria are unable to form spores, the acid-fast test can help categorize bacteria into smaller groups. Acid-fast organisms are characterized by wax-like, nearly impermeable cell walls, which contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids. Because their cell walls are so resistant to most of the compounds, acid-fast organisms require a special type of staining technique. The primary stain used in acid-fast staining, carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain to penetrate the cell wall. This is further assisted by adding heat. The smear is then rinsed with a very strong decolorizer, which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counter stain. If bacteria are positive for acid-fast they may be *Mycobacterium smegmatis*, and if bacteria are acid-fast negative they should be *Corynebacterium spp.* or *Lactobacillus spp*[14,15].

In the case of acid-fast negative, at first a catalase test should be carried out. If the results of the catalase test are positive, then the bacteria are most likely to be in the *Corynebacterium spp.* family; if the result is negative, then the bacteria are considered to be in the *Lactobacillus spp.* family.

To identify the bacteria from the above-mentioned family members, a starch hydrolysis (SH) test is carried out if the bacteria are from the *Corynebacterium spp.* family. TABLE 4 shows that the *Corynebacterium spp.* family is divided into two family members, one containing *Corynebacterium kutscheri*, which are SH-positive and the other containing *Corynebacterium xerosis*, which are SH-negative bacteria[15].

As previously mentioned, catalase-negative bacteria may belong to the *Lactobacillus spp.* family. To identify such bacteria, a glucose fermentation activity (GFA) test and a mannitol test should be carried out. If during the GFA test acid and gas are produced, the bacteria belongs to *Lactobacillus fermenti*; if the bacteria are only acid producers, they belong to the *Lactobacillus delbrueckii* or *Lactobacillus casei* strains[12].

The mannitol test can be a confirmatory test for identifying the bacteria. Bacteria are identified as *Lactobacillus casei* and *Lactobacillus delbrueckii*, if the

TABLE 4: Identification of all family members of *Corynebacterium spp.* and *Lactobacillus sp*

Corynebacterium spp. family	*Lactobacillus spp.* family
Corynebacterium kutscheri[1]	*Lactobacillus fermenti*[4]
Corynebacterium xerosis[2]	*Lactobacillus delbrueckii*[5]
	Lactobacillus casei

[1] Catalase positive, starch hydrolysis positive[2] Catalase positive, starch hydrolysis negative[4] Catalase negative, glucose fermentation positive, and gas productive[5] Catalase negative, mannitol negative, glucose fermentation positive, but not acid productive[6] Catalase negative, mannitol positive, glucose fermentation positive, but not acid productive

TABLE 5: Categorization of bacteria based on the starch hydrolysis test

Starch hydrolysis positive	Starch hydrolysis negative
B. subtilis	*B. laterosporus*
B. cereus	*B. pumilus (vp is pos., rest are neg.)*
B. megaterium	*B. marinus*
B. stearothermophilus	*B. sphaericus*
B. polymyxa	*B. schlegelii (grows at 55 °C, rest no)*
B. mycoides	*B. popilliae*
B. macerans	*B. pasteurii*
B. thuringiensis	*B. badius*
B. macquariensis	*B. lentimorbus*
B. licheniformis	*B. insolitus*
B. lentus	*B. azotoformans*
B. alvei	*B. larvae*
B. anthracis	
B. alcalophilus	
B. coagulans	
B. brevis	
B. circulans	
B. firmus	
B. pantothenticus	

mannitol test is positive and negative, respectively. All the above are summarized in TABLE 4.

IDENTIFICATION OF *BACILLUS SPP.* FAMILY MEMBERS

The first step to identify a *Bacillus spp*. family member is via the starch hydrolysis test, which classifies this family into two categories, as shown in TABLE 5. The purpose of the starch hydrolysis test is to determine whether bacteria are able to produce the alpha-amylase enzyme by using starch as a complex hydrocarbon from glucose. To perform this test, a small amount of bacteria is transferred to a culture medium containing starch. After overnight incubation, a few drops of iodine reagent are added to the culture medium to detect the presence of starch. The reaction occurring between the iodine reagent and the starch create a blue-black color in the culture medium. If a clear halo zone appears around the surrounding colonies, it indicates their ability to digest the starch present in the medium in the presence of alpha-amylase. As a result, the bacteria are considered to be starch hydrolysis positive[13,16].

TABLE 6: Categorization of bacteria based on catalase test

Catalase positive	Catalase negative
B. marinus	*B. popilliae*
B. insolitus	*B. azotoformans*
B. sphaericus	*B. larvae*
B. pasteurii	*B. pasteurii* [2]
B. laterosporus	
B. badius [1]	

[1] Spore is not round, rest are[2] Round spore, rest are oval

TABLE 7: Categorization of bacteria based on swollen cell test

Swollen cell positive	Swollen cell negative
B. sphaericus[1]	*B. insolitus*[2]
B. laterosporus	*B. marinus*[3]
B. pasteurii	

[1] Oval spore, rest are round[2] Acid from glucose negative[3] Acid from glucose positive

The next step is to categorize the bacteria as starch hydrolysis negative or starch hydrolysis positive.

Starch hydrolysis negative bacteria

As can be seen from TABLE 5, a large number of bacteria fall in each category, and it is necessary to classify them into smaller categories. Therefore, the catalase test can help to distinguish bacteria into two smaller categories, as seen in TABLE 6.

If the bacteria are catalase negative for two biochemical tests, namely the citrate and gelatinase tests, then the bacteria can be distinguished from each other. For example, through the citrate test, *B. azotoformans* can be distinguished because it is citrate positive. Secondly, if the citrate test proves negative, then the bacteria can either *B. larvae* or *B. popilliae*, of which *B. larvae* and *B. popilliae* are gelatinase positive and negative, respectively[11,16].

If the bacteria are determined catalase positive through the swollen cell test, they will be categorized into two categories as seen in TABLE 7.

If swollen cell positive cultures are able to produce oval-shaped spores, they are *B. sphaericus*, and if their spores are round, then the nitrate reduction test is called for. Nitrate broth is determines the ability of the bacteria to convert NO_3 to NO_2 using the enzyme nitrate reductase under anaerobic condition. After culturing the

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TABLE 8: Categorization of bacteria based on the VP test

Voges–Proskauer negative	*Voges–Proskauer positive*
B. coagulans	*B. circulans*
B. subtilis	*B. megaterium*
B. cereus	*B. stearothermophilus*
B. polymyxa	*B. macerans*
B. licheniformis	*B. badius*
B. mycoides	*B. pantothenticus*
B. thuringiensis	*B. macquariensis*
B. alvei	*B. lentus*
B. anthracis	*B. alcalophilus*
	B. brevis

bacteria in nitrate broth, if the color of nitrate broth changes from red to yellow, the result is positive. If the bacteria are positive for the nitrate reduction test, they are *B. pasteurii*. If they are nitrate reduction negative, they are identified as *B. sphaericus*[17].

In order to distinguish negative swollen cells from each other, the acid from glucose test can be used. The purpose of this test is to see whether the bacteria can ferment glucose as a carbon source. Phenol red glucose (dextrose) broth is used to perform the acid from glucose test. An inoculum from a pure culture of unknown bacteria is transferred aseptically to a sterile test tube containing dextrose. The inoculated tube is incubated at 35-37 C for 24 h and the results are determined. A positive test indicates a color change from red to yellow, indicating an acidic pH change[12,13,15].

Starch hydrolysis positive bacteria

Bacteria that show a positive result from starch hydrolysis analysis can be categorized into further smaller categories by using the Voges-Proskauer (VP) test (TABLE 8). The purpose of VP test is to determine that bacteria are able to produce non-acidic or neutral end products. To perform the VP test, first bacteria are transferred into a tube containing VP broth and incubated overnight. Then a regent containing a mixture of 60% alpha-naphthol and 40% potassium hydroxide is added to the tube. After adding the reagent a cherry red color appears, indicating that the VP test result is positive. If a yellow-brown color appears, it demonstrates a negative result for the VP test[18].

(a) Voges-Proskauer positive bacteria

(A) Bacteria with diameter e" 1 ìm

TABLE 9: Bacteria with different diameters

Diameter ? 1 µm	*Diameter < 1 µm*
B. cereus[1]	*B. alvei*
B. anthracis[2]	*B. subtilis*
B. thuringiensis[3]	*B. polymyxa*
B. mycoides[4]	*B. licheniformis*
	B. coagulans

[1] Motile [2] Nonmotile [3] Makes insecticidal protein
[4] Colony with rhizoidal appearance

Microscopic observation should be conducted to determine the bacterial diameter for VP positives. Based on this observation, VP positives can be divided into bacteria with a diameter greater than or equal to 1 ìm and bacteria with a diameter less than 1 ìm (TABLE 9).

Four bacteria are categorized with a diameter of e" *1* ì*m*, namely *B. cereus, B. anthracis, B. thuringiensis*, and *B. mycoides*. Many characteristic features such as motility, making of insecticidal protein, and colony morphology can be used to distinguish these bacteria. If bacteria with diameter equal or greater than 1 µ*m* are motile, they are identified to be *B. cereus;* if nonmotile, they are *B. anthracis*. Furthermore, if bacteria are able to product insecticidal protein, they are *B. thuringiensis*, commonly applied as a biological pesticide. *B. thuringiensis* can produce a crystal protein called δ-endotoxin, which also has insecticidal action[18].

(B) Bacteria with diameter greater than 1 ìm

On the other hand, if bacteria are categorized into the segment of bacteria with a diameter less than 1 ì*m* (TABLE 9), the citrate test can be employed to create a smaller category, as shown in TABLE 10.

Citrate negative bacteria are limited to *B. polymyxa* and *B. alvei*. The mannitol fermentation test (MFT) is generally applied to distinguish these two strains from each other. If the bacteria are MFT positive or negative, they are *B. polymyxa* or *B. alvei*, respectively. The purpose of MFT is to detect bacteria that have the

TABLE 10: Categorization of bacteria based on citrate test

Citrate positive	Citrate negative
B. subtilis	*B. polymyxa* [1]
B. coagulans	*B. alvei* [2]
B. licheniformis	

[1]*Mannitol* fermentation *test* positive[2] *Mannitol* fermentation *test* negative

TABLE 11: Categorization of VP positive bacteria based on the swollen cell test

Swollen cell positive bacteria	Swollen cell negative bacteria
B. stearothermophilus [1]	*B. megaterium* [3]
B. macerans	*B. badius*[4]
B. pantothenticus	
B. macquariensis	
B. lentus [2]	
B. alcalophilus	
B. brevis	
B. circulans	

[1] Growth at 55°. The rest of the bacteria in the column of this table cannot grow in this temperature. [2] Catalase negative. The rest of the bacteria in the left column of this table are catalase positive. [3] Citrate positive [4] Citrate negative

ability to use mannitol as their carbon source. To perform MFT, bacteria are added to a sterile tube containing phenol red mannitol broth and then incubated for 24 h. The change of color from red to yellow means the results of the MFT are positive, and if there is no color change, the result is considered negative[19].

Based on TABLE 10, citrate-positive bacteria are *B. subtilis*, *B. coagulans*, and *B. licheniformis*. These bacteria can be categorized further by determining their growth ability in a medium containing 6.5% sodium chloride (NaCl). To determine this growth ability, bacteria are added to a sterile test tube containing 6.5% NaCl broth (which is a mixture of nutrient broth and 6.5% NaCl) and incubated for 24 h. A positive test is indicated by the presence of turbidity. Bacteria that grow in a medium containing 6.5% NaCl are *B. subtilis* and *B. licheniformis*. These bacteria are easily distinguished from each other by testing their ability to grow at 55 °C. If the bacteria are able to grow at 55 °C, they are identified to be as *B. licheniformis*; otherwise, they belong to *B. subtilis*[17,19].

(b) Voges-Proskauer negative bacteria

VP-negative bacteria can be divided into two categories by using the swollen cell test, as shown in TABLE 11.

Swollen cell positive bacteria can be divided into smaller categories by testing their ability to grow in a culture medium containing 6.5% NaCl (TABLE 12)[14].

A bacterium unable to grow in 6.5% NaCl belongs to *B. pantothenticus* and *B. circulans*, which can be distinguished by using the arabinose fermentation test

TABLE 12: Categorization of swollen cell positive bacteria by testing their ability grow in 6.5% NaCl

Bacteria with the ability to grow in 6.5% NaCl	Bacteria without the ability to grow in 6.5% NaCl
B. circulans	*B. pantothenticus* [1]
B. macerans	*B. circulans* [2]
B. macquariensis	
B. alcalophilus	
B. brevis	

[1] Acid via arabinose negative [2] Acid via arabinose positive

TABLE 13: Categorization of bacteria based on their ability to grow in 6.5% NaCl with AFT

AFT positive	AFT negative
B. macquariensis [1]	*B. macerans* [3]
B. brevis [2]	*B. alcalophilus* [4]
	B. circulans [5]

[1] Methyl red test positive [2] Methyl red test negative [3] Gas from glucose positive [4] Gas from glucose negative and no growth under pH<7.0 [5] Gas from glucose negative and growth under pH < 7.0

(AFT). The purpose of the AFT is to indicate which bacteria can use arabinose as their carbon source. Phenol red arabinose broth, a mixture of nutrient broth and 0.5 to 1% arabinose, can be used for AFT. To perform AFT, bacteria are transferred to a sterile tube containing phenol red arabinose broth and incubated for 24 h. In the case of a positive result, the color changes red to yellow[11,12].

In addition, to distinguish bacteria that can grow in 6.5% NaCl, AFT can be applied. Based on AFT, the bacteria can be divided into two categories (TABLE 13).

AFT-positive bacteria containing *B. macquariensis* and *B. brevis* can be distinguished via the methyl red test (MRT). If the bacteria are MRT positive, they are identified as *B. macquariensis*, and if the bacteria are MRT negative, they are identified as *B. brevis* (TABLE 13).

AFT-negative bacteria containing *B. macerans*, *B. alcalophilus*, and *B. circulans* can be also distinguished based on gas from the glucose test. When bacteria ferment glucose, they produce gases, mainly carbon dioxide and hydrogen. These gases bubble up through the medium and escape into the atmosphere. Tubes of broth media can be made with inverted tubes called Durham tubes, which are filled with the medium after sterilization. Durham tubes serve as gas traps for

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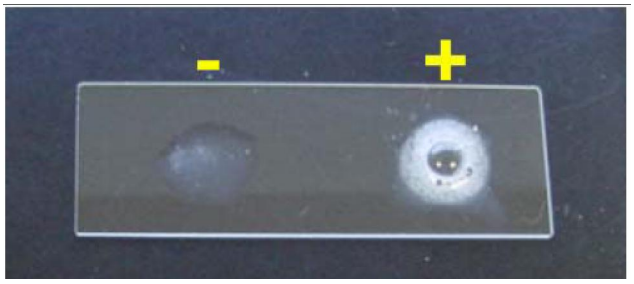

Figure 4: Releasing oxygen bubbles when bacteria are able to produce the catalase enzyme (Source: Microbiology Lab Tutorial)

TABLE 14: Categorization of gram-positive cocci bacteria based on catalase test

Catalase positive	Catalase negative
Micrococcus spp.	*Streptococcus spp.*
Staphylococcus spp.	*Streptococcus spp.*
	Streptococcus pneumoniae
	Streptococcus pyogenes
	Enterococcus spp.
	Streptococcus mitis

collecting bubbles generated by gas-producing microbes. Dextrose can be used to perform this test and gas collected in Durham tubes means the test is positive[15].

GRAM-POSITIVE COCCI

As it can be seen from TABLE 1, Gram-positive bacteria can be divided into two categories based on their morphology (cocci and bacillus). In this part of the methodology, distinguishing Gram-positive cocci is discussed. First, the bacteria selected to undergo the catalase test are divided into two categories (TABLE 14). The purpose of the catalase test is to determine the ability of bacteria to product the catalase enzyme. The catalase enzyme can decompose hydroxyl peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). Bacteria are catalase-positive if oxygen bubbles are observed when a small amount of bacterial suspension is added to the hydrogen peroxide (Figure 4)[18].

Catalase-positive bacteria

Catalase-positive bacteria, shown in TABLE 15, can be distinguished from each other by using MFT. MFT's details are explained in Section 5.2.1.2. Using MFT, the catalase-positive bacteria in TABLE 14 can

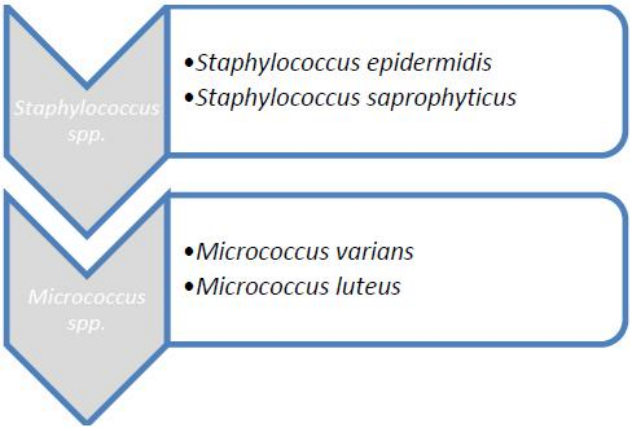

Figure 5: Subfamilies of *Micrococcus spp.* and *Staphylococcus spp*.

TABLE 15: Categorization of catalase-positive bacteria based on MFT

MFT positive	MFT negative
Staphylococcus aureus	*Staphylococcus spp.*
	Micrococcus spp.

be divided into two parts (TABLE 15). TABLE 15 identifies the only remaining bacterial strain that is MFT positive as *Staphylococcus aureus*. *Staphylococcus spp*. and *Micrococcus spp*. are MFT-negative, and can be distinguished by other tests such as the pigment color test. In the pigment color test, bacteria are inoculated into the nutrient agar culture medium, and after 24 h incubation, colony color can be observed. If the bacteria produce yellow pigment, they belong to the *Micrococcus spp*. family; otherwise, they are from the *Staphylococcus spp*. family. In fact, each has two family members, i.e., *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* from the *Staphylococcus spp*. family, and *Micrococcus varians* and *Micrococcus luteus* from the *Micrococcus spp*. family (Figure 5)[19].

The *Staphylococcus spp*. family has two members, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. To distinguish these two family members, the novobiocin susceptibility test (NST) can be applied. The NST determines whether bacteria are sensitive (susceptible) to novobiocin or resistant to the drug. Blood agar medium or nutrient agar medium can be used in performing the NST. Bacteria are aseptically spread over a sterile plate containing one of the above-mentioned media, and an antibiotic-impregnated disk containing novobiocin is then aseptically placed in

Figure 6: The left side is a bacterium resistant to novobiocin, while on the right is a bacterium susceptible to novobiocin (Source: http://www.studyblue.com).

Figure 7: Differences among alpha, beta and gamma bacteria after growing the bacteria in blood agar media (Source: http://www.studyblue.com)

the center of the agar surface. The plate is incubated at 35-37 °C for 24 h and the results are determined. Growth of the bacterial lawn up to the margin of the disk indicates the bacteria are resistant to the antibiotic. A clear zone around the disk, termed the zone of inhibition, indicates the bacteria are susceptible to the antibiotic. If bacteria are susceptible to novobiocin, they are identified as *Staphylococcus epidermidis*. If the bacteria are not resistant against novobiocin, they are identified to be *Staphylococcus saprophyticus* (Figure 6)[15].

As explained in Figure 2, *Micrococcus spp*. has two family members named *Micrococcus varians* and *Micrococcus luteus*. GFA can be used to distinguish them from each other. Details of GFA are explained in Section 4.2[12,14].

Catalase-negative bacteria

As can be seen from TABLE 14, bacteria can be divided into catalase-positive bacteria and catalase-negative bacteria. In this section, the identification of catalase-negative bacteria is discussed. The first step is to identify catalase-negative cocci bacteria based on the application of the hemolysis test, which divides the bacteria into three categories, alpha (α), beta (β), and gamma (γ) (TABLE 16)[18].

The hemolysis test provides information on whether the bacterium possesses hemolytic enzymes. By providing a culture medium enriched with red blood cells, it is possible to determine whether the bacterium is able to destroy the cells and whether it can digest the hemoglobin present inside. To perform the hemolysis test, bacteria are transferred on a plate containing blood agar medium, which is incubated overnight. If the medium is discolored or found darkened after growth, the organism has demonstrated alpha-hemolysis. If the medium appears clear after growth, the organism is known to be as beta-hemolytic. If there is no discernible change in the medium's color, the bacteria tested are said to be non-hemolytic (Figure 7)[17,18].

TABLE 16: Categorization of catalase-negative cocci bacteria using hemolysis test

Alpha (α)	Beta (β)	Gama (γ)
Streptococcus pneumoniae[1]	*Streptococcus pyogenes*	*Enterococcus spp.*
Streptococcus mitis[2]	*Streptococcus spp.*	*Streptococcus spp.*

[1] Optochin sensitivity test is positive [2] Optochin sensitivity test is negative

(a) Alpha bacteria

As seen from TABLE 16, *Streptococcus pneumoniae* and *Streptococcus mitis* are alpha bacteria. To distinguish between these two strains, the optochin sensitivity test (OST) should be applied. If the bacteria are OST positive, they belong to *Streptococ-*

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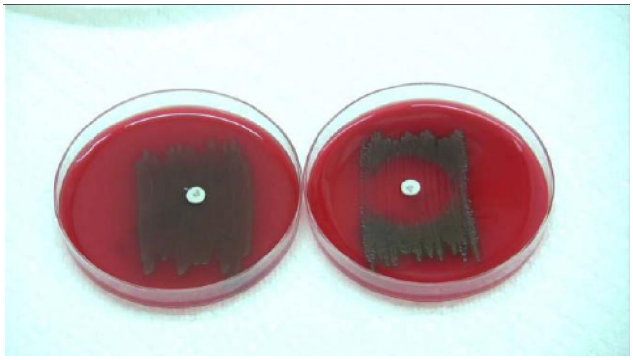

Figure 8: The left side shows bacteria that are resistant to optochin. The right side shows bacteria susceptible to optochin (Source: optochin test for *Streptococcus pneumoniae* from a YouTube video)

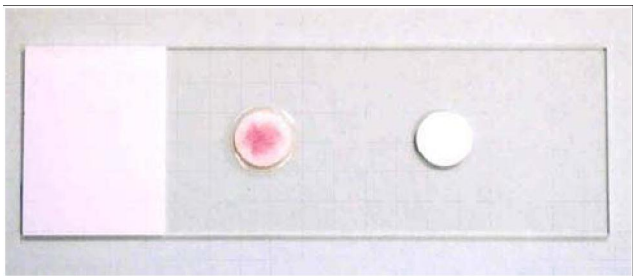

Figure 9: Right side shows a positive result for a PYR test, while the left side displays a negative result

cus pneumoniae. If the bacteria are OST negative, they are identified as *Streptococcus mitis*. Optochin is a chemical that can be toxic for some bacteria but harmless to others. This test is applied to identify *Streptococcus pneumoniae*, which is most commonly susceptible to Optochin. To perform OST, first bacteria are transferred onto a plate containing nutrient agar. Next, a optochin-impregnated disk is placed on the center of nutrient agar medium. Then the plate is incubated overnight. If the bacteria are susceptible to optochin, there will be a visible zone of inhibition formed around the disk, representing the area where chemical concentration has prevented bacterial growth (Figure 8)[11,16].

(b) Beta bacteria

Beta bacteria containing *Streptococcus pyogenes* and *Streptococcus spp*. can be distingushed from each other by using the pyrrolidone arylamidase (PYR) test. If bacteria are PYR positive, they are identified as *Streptococcus pyogenes*, and if bacteria are PYR negative, they are *Streptococcus spp* (*Streptococcus agalactiae*). The purpose of this test is to determine the activity of the pyrrolidonyl arylamidase enzyme in the bacteria. A small amount of bacteria is transferred onto a paper disk and impregnated with pyrrolidonyl arylamidase. Changing color to bright pink or cherry red is a positive result; otherwise it is negative (Figure 9)[15].

TABLE 17: Categorization of gamma bacteria based on the BEA test

BEA negative	BEA positive
Streptococcus spp.	*Enterococcus faecalis*
	Enterococcus mundtii
	Enterococcus faecium
	Enterococcus hirae

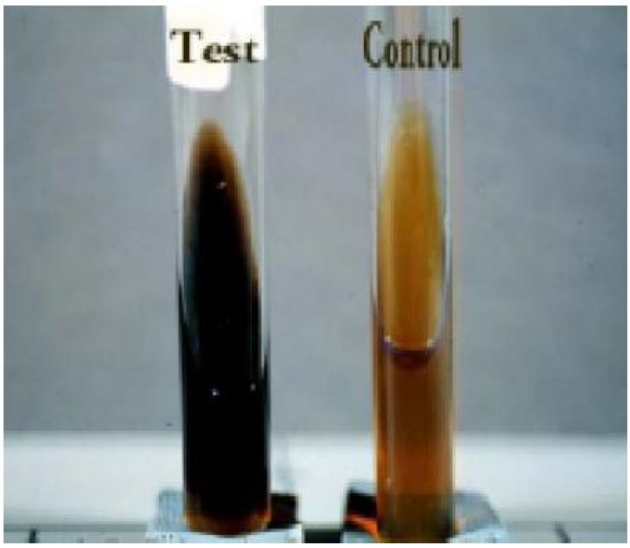

Figure 10: Left tube shows a positive result for the BEA test. The right displays a negative result for this test. (Source: http://www.studyblue.com)

(c)Gamma bacteria

Gamma bacteria containing *Enterococcus spp*. and *Streptococcus spp*. can be distinguished by the bile esculin agar test (BEA test). The purpose of the BEA test is to see whether bacteria are able to hydrolyze esculin as their carbon source. In the BEA test, a small amount of bacteria are aseptically transferred to a sterile test tube containing bile esculin agar. The tube is then incubated at 35-37 °C for 24 h. If the bacteria grow in the medium, a chocolate brown color appears and the test is considered positive; otherwise, it is negative (Figure 10). Based on the BEA test, gamma bacteria are divided into two groups, as shown in TABLE 17[13,19].

BEA-positive bacteria can be distinguished by using the potassium tellurite (PT) test, as shown in TABLE 18. To perform the PT test, a small amount of bacteria

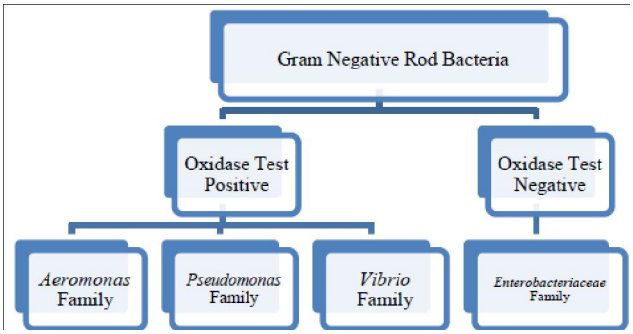

Figure 11: Categorization of Gram-negative rod bacteria based on the oxidase test

TABLE 18: Categorization of BEA positive bacteria based on the PT test

PT test positive	PT test negative
Enterococcus faecalis	*Enterococcus faecium*[1]
	Enterococcus hirae[2]
	Enterococcus mundtii[3]

[1] Mannitol positive., white colony [2] Mannitol negative [3] Mannitol positive., yellow colony

are transferred on a plate containing potassium tellurite agar. The plate is incubated for 24 h. Bacteria that are positive for the PT test reduce the potassium tellurite present in the agar, which in turn makes the colonies grow black[19].

Enterococcus faecium, *Enterococcus hirae*, and *Enterococcus mundtii* can be distinguished via the mannitol test, given the color of their colonies (TABLE 18). Details about the mannitol test are explained in Section 5.2.1.2.

GRAM-NEGATIVE ROD BACTERIA

Gram-negative rod-shaped bacteria divide into two large families with the help of the oxidase test, as detailed in Figure 11.

Figure 11 shows that gram-negative rod bacteria are divided into two categories by the oxidase test: 1) *Aeromonas*, *Pseudomonas* and *Vibrio* families, and 2) the *Enterobacteriaceae* family. With continuous methods of investigation, the three families containing the *Aeromonas*, *Pseudomonas* and *Vibrio* families are explained (part of Section 7.1). In addition, *Enterobacteriaceae* is a very large family, separately discussed in Section 7.2[12].

Oxidase test positive bacteria (Aeromonas, Pseudomonas and Vibrio families)

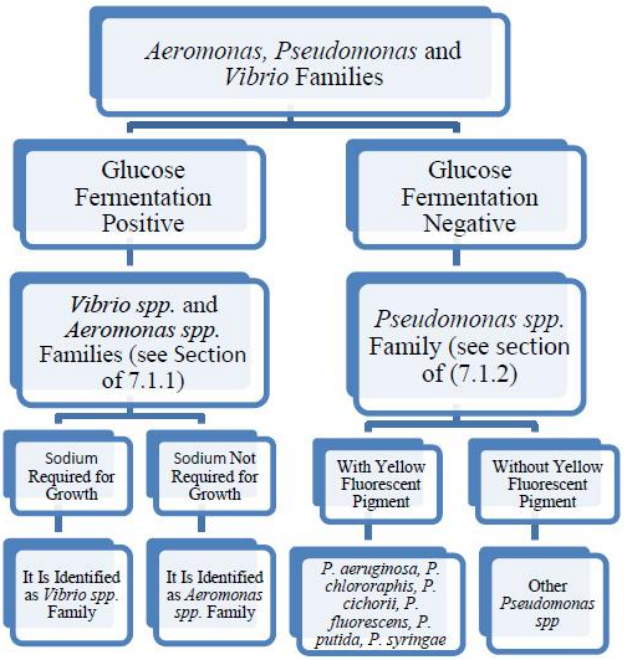

Figure 12: Categorization of *Aeromonas, Pseudomona* and *Vibrio* families based on glucose fermentation test

Aeromonas, *Pseudomonas* and *Vibrio* families can be distinguished with the help of the glucose fermentation test. If this test is positive, bacteria are from the *Aeromonas* and *Vibrio* families. However, if the Glucose fermentation test is negative, bacteria are from *Pseudomonas spp*. (Figure 12)[18].

It should be noted that the purpose of the glucose fermentation test is to determine whether bacteria can ferment glucose as their main carbon source. To perform this test, a small amount of bacteria is transferred aseptically to a sterile test tube containing dextrose. The tube in this test is a Durham tube, a small inverted tube that can serve as a trap for gas bubbles generated during the fermentation of sugars. The inoculated tube is incubated at 35-37 C for 24 h. A positive test consists of a color change from red to yellow, indicating a pH change toward the acidic side (Figure 13)[16,17].

(a) Vibrio spp. and Aeromonas spp. families

These families can be distinguished by determining the ability of the bacteria to grow with or without the sodium ion.

(A) Bacteria that require sodium for growth

If bacteria can grow without sodium, they are identified in the *Vibrio* family; otherwise, they are in the *Aeromonas spp*. family. The *Aeromonas spp*. family

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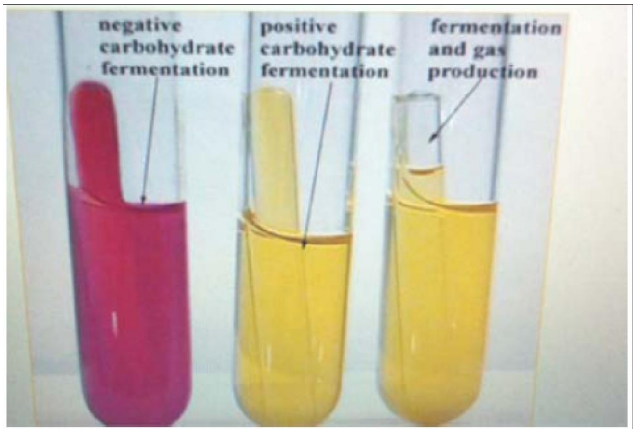

Figure 13: Possible results of glucose fermentation test. 1) In the left part of the figure, the tube is red and shows a negative result. 2) In the center of the figure, the tube color has changed from red to yellow without gas production, which shows a positive result without gas production. 3) In the right part of the above figure, tube color has changed from red to yellow and gas is produced, showing a positive result with gas production. (Source: http://www.studyblue.com)

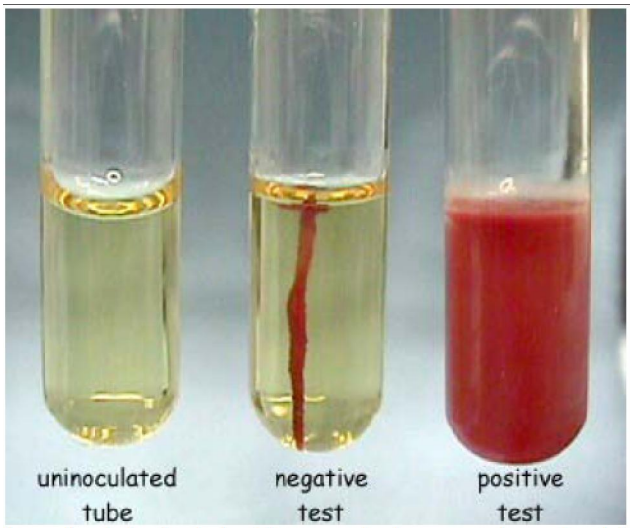

Figure 14: Left side tube shows motility medium before incubation. The center tube shows a negative result and right side tube shows a positive result. (Source: http://old.infectionnet.org/)

can be distinguished from each other by using the VP test. Details of this test are in the previous section (TABLE 19)[12].

VP-positive bacteria (TABLE 18) can be divided into smaller categories by using the motility test, shown in TABLE 19.

For making Motility test medium a mixture of 3g beef extract, 10g pancreatic digest of casein, 5g sodium chloride, 4g agar-agar and 1 liter distillated water is used for the preparation of the medium.. Then 5 ml of

TABLE 19: Categorization of *Aeromonas spp.* family with the VP test

VP positive	VP negative
A. hydrophila[2]	Other *Aeromonas spp.*
A. veronii[2]	
A. sobria[1]	
A.salmonicida (Subsp .masoucida3)[3]	

[1] No gas from glucose [2] Motile [3] Nonmotile

triphenyltetrazolium chloride (TTC) is added. TTC is a part of this formulation, as bacteria can reduce it and create a diffuse red color that visualizes bacterial motility (Figure 14). To test for motility, a sterile needle is inoculated with pure bacterial culture and is stabbed into the test tube containing the motility medium (Figure 14) to within 1 cm of the bottom of the test tube. The needle must be in the same line as it enters and is removed from the motility medium. Then the test tube is incubated at 35 °C for 24 h or until growth is evident, per Figure 14. A positive motility test is indicated by a red turbid area extending away from the line of inoculation. A negative test is indicated by red growth along the inoculation line but no further (Figure 14)[11,17,19].

Motile bacteria in TABLE 19 can be distinguished from each other through the H_2S test. The purpose of the H_2S test is to determine whether the bacteria are able to convert sulfur to sulfides during their metabolism. If bacteria are H_2S positive, they are identified as *A. hydrophila*; if they are H_2S negative, they are identified as *A. veronii*. To perform the H_2S test, bacteria should be aseptically transferred onto a sterile test tube containing triple sugar iron agar (TSIA). The tube should be incubated at 35-37 °C for 24 h. The iron ions (Fe^{2+}) present in TSIA have a strong attraction for sulfide ions. The result is that H_2S combines with iron to make FeS, a black-colored compound. In the tubes of TSIA containing bacteria producing hydrogen sulfide, agar turns black from the FeS and the result is positive (Figure 15)[18,19].

(B) Bacteria that do not require sodium to grow

The *Vibrio spp*. family can be distinguished from the *Aeromonas spp*. family by testing its ability to grow in the presence or absence of the sodium ion. Bacteria that can grow without sodium are from the *Vibrio spp*.

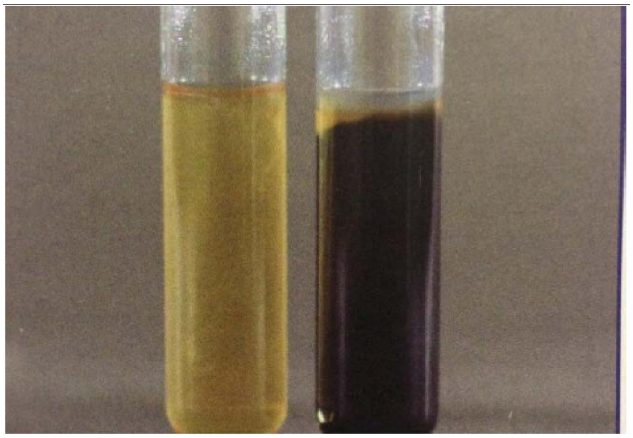

Figure 15: Right-side tube shows negative result for H_2S test, while the left one shows a positive result for this test. (Source: http://www.studyblue.com)

TABLE 22: Categorization of *Pseudomonas spp.* family using the fluorescent pigment test

With yellow fluorescent pigment	Without yellow fluorescent pigment
P. syringae [1]	Other *Pseudomonas spp.*
P. aeruginosa	
P. chlororaphis	
P. cichorii	
P. fluorescens	
P. putida	

[1] Oxidase negative

family. The *Vibrio spp*. family can also be distinguished from each other via the luminescent test. Bacteria can produce a special substance called autoinductor, which during their growth and multiplication is accumulated in their environment. If amount of autoinductor goes higher than a critical level, it can induce the luciferase enzyme to generate a luminous emission[3,4]. This luminous emission can be detected with a luminometer. Based on luminescent test, *Vibrio spp*. can be divided into two categories as shown in TABLE 20[15].

Luminescent-positive bacteria can be divided into two small categories by testing their pigments. If they have yellow pigment, they are from the *V. fischeri* or *V. logei* strain. If they do not have any pigment or their pigment is not yellow, they are from the *V. orientalis* or *V. splendidius* strains (TABLE 21)[14].

Finally, the bacteria in TABLE 21 can be distinguished by testing their ability to grow at 30 and 37 °C (TABLE 21).

(b) *Pseudomonas spp*. family

Some *Pseudomonas spp*. Families produce diffusible, fluorescent pigments on pseudomonas F agar medium, and this characteristic can be used to categorize *Pseudomonas spp.* families into two groups, 1) with yellow fluorescent pigment, and 2) without fluorescent yellow pigment (TABLE 22)[12].

To performed a diffusible, fluorescent pigments test, a pure culture of bacteria is transferred onto a plate containing pseudo F agar medium. The plate should be incubated at 25 ºC. The developed bacterial cultures can be examined with the help of UV light[7-10]. A positive result is the observance of a greenish-yellow pigment in the pseudo F agar medium that fluoresces under UV light (Figure 16).

TABLE 20: Categorization of bacteria based on luminescent test

Luminescent positive	Luminescent negative
V. splendidius	Other *Vibrio spp.*
V. fischeri	
V. logei	
V. orientalis	

TABLE 21: Categorization of luminescent positive bacteria by the pigment test

Yellow pigment	Non-pigment or other pigment color
V. fischeri [1]	*V. orientalis* [3]
V. logei [2]	*V. splendidius* [4]

[1] Oxidase negative

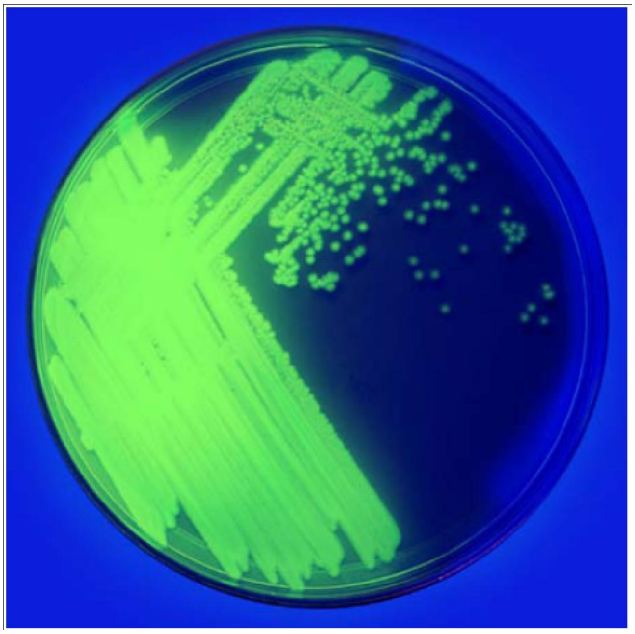

Figure 16: A greenish-yellow pigment on the pseudo F agar medium under UV light (Source: www.thermoscientific.com)

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TABLE 23: Categorization of bacteria with yellow fluorescent pigment based on the nonfluorescent diffusible blue pigment test

Without blue nonfluorescent pigment	With blue nonfluorescent pigment
P. syringae	*P. aeruginosa*
P. chlororaphis	
P. cichorii	
P. fluorescens	
P. putida	

TABLE 24: Categorization of bacteria without blue nonfluorescent pigment by the nitrate reduction test

Nitrate reduction positive	Nitrate reduction negative
P. chlororaphis [1]	*P. putida* [3]
P. fluorescens [2]	*P. syringae* [4]
	P. cichorii [5]

[1] Colony pigmented [2] To differentiate biovars, perform a lecithinase test [3] Lecithinase negative [4] Oxidase negative [5] Lecithinase positive

The nonfluorescent diffusible pigment test can be used to identify the division of bacteria with yellow fluorescent pigment (TABLE 20) into two categories (TABLE 23). The purpose of the nonfluorescent diffusible pigment test is to determine the bacteria's ability to produce pyocyanin, a blue compound. For this test, *Pseudomonas* agar P medium should be used. This medium enhances the production of pyocyanin by the addition of magnesium chloride and potassium sulfate, and inhibits the formation of fluorescein. The appearance of blue colonies on *Pseudomonas* agar P medium shows a positive result[11].

Finally, with the nitrate reduction, lecithinase, pigment and oxidase tests, all *Pseudomonas* family members can be distinguished from each other (TABLE 24).

In the previous sections, the methods of colony pigment and nitrate reduction tests have been explained. However, the lecithinase test has not yet been discussed, and so now it is discussed further here. To perform the lecithinase test, a small amount of bacterial culture is aseptically transferred to a sterile plate containing egg yolk agar medium and incubated at 35 °C for 24-48 h. A positive result is obtained for a lecithinase test when an opaque halo appears surrounding the colonies (Figure 17)[12].

Oxidase test negative bacteria (Family *Enterobacteriaceae*)

Figure 17: Left side shows an opaque halo surrounding colonies, which means a positive result for this lecithinase test. The right side shows a negative result. (Source: http://pictures.life.ku.dk)

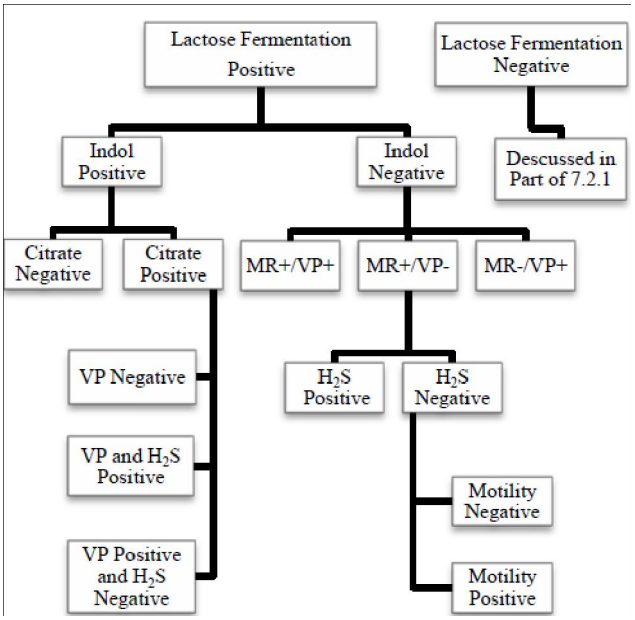

Figure 18: Identification chart for *Enterobacteriaceae* family with a positive lactose fermentation test

The *Enterobacteriaceae* family can be divided into two large families based on lactose fermentation. These families are called lactose fermentation positive and lactose fermentation negative. Figure 18 shows biochemical tests that should be used for the identification of the *Enterobacteriaceae* family[14,17].

(a) Lactose fermentation positive bacteria

Based on the lactose fermentation test, the *Enterobacteriaceae* family is divided into two large categories containing lactose fermentation positives and negatives. TABLE 25 displays all family members of *Enterobacteriaceae* that are positive for lactose fermentation[19].

The bacteria in TABLE 25 are divided into two

TABLE 25: List of bacteria that are positive for lactose fermentation

Lactose fermentation positive	
Enterobacter intermedius	*Serratia rubidaea*
Citrobacter diversus	*Erwinia carotovora*
Citrobacter freundii	*Erwinia chrysanthemi*
Enterobacter aerogenes	*Escherichia coli*
Enterobacter cloacae	*Klebsiella oxytoca*
Enterobacter amnigenus	*Klebsiella pneumonia (subsp. Ozaenae and Pneumoniae)*
	Serratia fonticola

TABLE 26: Categorization of lactose fermentation positive bacteria based on the indole test

Indole-positive bacteria	Insole-negative bacteria
Citrobacter diversus	*Enterobacter intermedius*[1]
Escherichia coli	*Serratia rubidaea*[3]
Erwinia chrysanthemi	*Erwinia carotovora*[3]
Klebsiella oxytoca	*Citrobacter freundii*[2]
	Serratia fonticola[2]
	Klebsiella pneumonia (Subsp. Ozaenae)[2]
	Klebsiella pneumonia (Subsp. Pneumoniae)[3]
	Enterobacter aerogenes[3]
	Enterobacter cloacae[3]
	Enterobacter amnigenus[3]

[1]Both MR and VP tests are positive
[2]MR and VP tests are positive and negative, respectively
[3]MR and VP test are negative and positive, respectively

groups by the indole test, shown in TABLE 26. Furthermore, based both MR and VP test bacteria in TABLE 26 can be divided again into a third category containing MR+/VP+, MR+/VP- and MR-/VP+ (TABLE 26)[12].

The MR test or methyl red test determines whether bacteria are able to perform mixed acid fermentation in the presence of glucose. If bacteria are able to perform mixed acid fermentation in presence of glucose, they can produce a variety of acids, which can be significantly decreased in the pH of the medium. Addition of a pH indicator such as methyl red, which changes color below pH 4, indicates the presence of extreme acidity associated with mixed acid fermentation. To perform this test, a small amount of bacterial culture should be aseptically transferred to a sterile test tube containing MR-VP broth. Then the tube should be incubated at 35-37 °C for 24 h. After incubation, five drops of methyl red are added to the incubated tube. If the color changes from yellow to red it is a positive result and if not, the result is negative[16].

TABLE 27: Categorization of MR+ and VP- bacteria based on the H_2S test

H_2S positive	H_2S negative
Citrobacter freundii	*Serratia fonticola*[1]
	Klebsiella pneumoniae (Subsp. Ozaenae)[2]

[1]Motility positive [2]Motility negative

Bacteria in TABLE 26, which are MR+ and VP negative, can be divided into two smaller categories through the H_2S production test (TABLE 27). As can be seen from TABLE 27, all bacteria can be distinguished from each other with the motility test[18].

Bacteria that are MR- and VP+ can be distinguished from each other by using the decarboxylase, pigment, motility, and sorbitol fermentation tests (TABLE 28).

The purpose of the lysine decarboxylase test is to determine whether bacteria are able to use the amino acid lysine as their carbon and energy source. To per-

TABLE 28: Categorization of MR- and VP+ using the lysine decarboxylase test

Lysine decarboxylase positive	Lysine decarboxylase negative	Lysine decarboxylase, no result
Klebsiella pneumoniae Subsp. pneumoniae[1]	*Enterobacter Cloacae*[4]	*Erwinia carotovora*[6]
Serratia rubidaea[2]	*Enterobacter Amnigenus*[5]	
Enterobacter aerogenes[3]		

[1] Nonmotile, no pigment [2] Motile, red pigment [3] Motile, no pigment [4] Acid from sorbitol [5] No acid from sorbitol
[6] Lysine unknown but gelatinase positive; none above are positive

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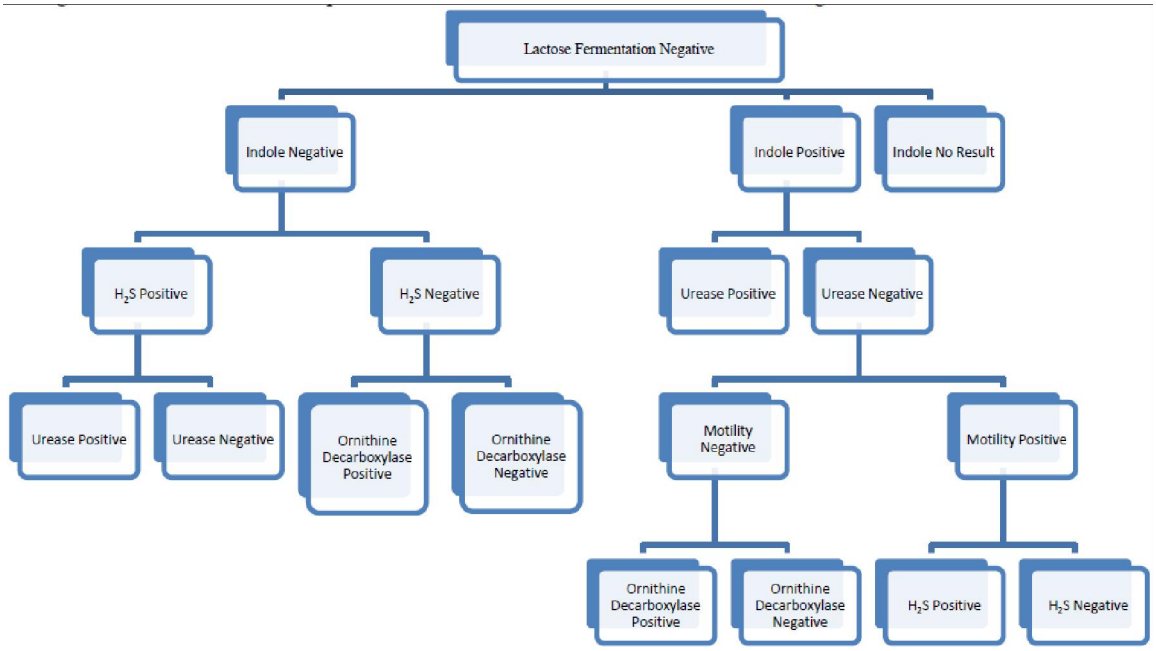

Figure 19: Identification chart of *Enterobacteriaceae* family with negative lactose fermentation test

form a decarboxylase test, a small amount of a pure culture of bacteria should be aseptically transferred to a test tube containing lysine decarboxylase broth, which is a mixture of nutrient broth and 5% lysine. Then the tube is incubated at 35-37 °C for 24 h. During incubation, most of the bacteria use glucose, which causes the pH to drop. Therefore, in an acidic condition the color of the pH indicator, bromocresol purple, is changed from purple to yellow. In an acidic environment, the enzyme lysine decarboxylase is activated. The tube should be incubated for additional 24 h in the same condition to allow bacteria to use the amino acid lysine. If the color of medium changes back to yellow after an additional 24 h, it indicates a positive result. If not, the result of this test is considered negative. In addition, if the bacteria are not able to use glucose, there is no result[13].

The purpose of the sorbitol fermentation test is to determine whether bacteria are able to use the carbohydrate sorbitol as their carbon source or not. If bacteria have the ability to use sorbitol, they will produce an acid end product, which causes a decline in the pH of the medium. To indicate a pH decline, a pH indicator such as phenol red can be applied. To perform a sorbitol test, a small amount of pure bacteria should be aseptically transferred to a sterile tube of phenol red sorbitol broth, which is a mixture of nutrient broth and 0.5 to 1% sorbitol. The tube should be incubated at 35-37 °C for 24 h. A positive result changes the color from yellow to red[12,13].

TABLE 29 : List of bacteria that are lactose fermentation negative

Lactose fermentation negative	
Edwardsiella tarda	
Erwinia cacticida	*Serratia marcescens*
Morganella morganii	*Serratia liquefaciens*
Proteus mirabilis	*Shigella spp. (boydii, dysenteriae, flexneri)*
Proteus penneri	*Shigella sonnei*
Proteus vulgaris	*Yersinia enterocolitica*
Providencia stuartii	*Yersinia pestis*
Salmonella bongori	*Yersinia pseudotuberculosis*
Salmonella enterica	

Another test that should be used for distinguishing bacteria is shown in TABLE 28, the gelatin test. The purpose of the gelatin test is to determine whether bacteria are able to produce the gelitinase enzyme in order to utilize the protein gelatin as their carbon source. To perform a gelatinase test, a small amount of pure bacterial culture should be aseptically transferred to a sterile test tube containing gelatin agar, which is a mixture of nutrient agar and 15% gelatin. A positive result is declared when the form of the medium changes from semisolid to liquid, so that it cannot solidify again[13].

(a) Lactose fermentation negative bacteria

Figure 19 shows all the tests required for the identification of lactose fermentation negative bacteria.

By using the indole test, the bacteria in TABLE 29 are divided into three groups, shown in TABLE 30.

TABLE 30: Categorization of lactose fermentation negative bacteria using the indole test

Indole positive	Indole negative	Indole no result
Edwardsiella tarda	*Yersinia pseudotuberculosis*	*Shigella spp.* *(boydii, dysenteriae, flexneri)*
Morganella morganii	*Proteus mirabilis*	*Yersinia enterocolitica*
Proteus vulgaris	*Proteus penneri*	
Providencia stuartii	*Erwinia cacticida*	
	Salmonella bongori	
	Salmonella enterica	
	Serratia marcescens	
	Serratia liquefaciens	
	Shigella sonnei	
	Yersinia pestis	

TABLE 31: Categorization of indole-positive bacteria by using the H_2S test

H_2S-positive bacteria	H_2S-negative bacteria
Edwardsiella tarda[1]	*Morganella morganii*[3]
Proteus vulgaris[2]	*Providencia stuartii*[4]

[1] Urease test positive [2] Urease test negative [3] Ornithine decarboxylase negative [4] Ornithine decarboxylase positive

TABLE 33: Categorization of urease-negative bacteria by the motility test

Positive motility test bacteria	Negative motility test bacteria
Erwinia cacticida[1]	*Shigella sonnei*[6]
Salmonella bongori[2]	*Yersinia pestis*[7]
Salmonella enterica[3]	
Serratia marcescens[4]	
Serratia liquefaciens[5]	

[1] Lysine decarboxylase test negative bacteria and the rest of the bacteria in the left column of this table are lysine decarboxylase test positive. [2] H_2S positive, KCN growth test positive [3] H_2S positive, KCN growth test positive [4] H_2S negative, ability to produce red pigment [5] H_2S negative, no pigment production ability [6] Ornithine decarboxylase test positive [7] Ornithine decarboxylase test negative

(A) Indole-positive bacteria

Indole-positive bacteria including *Edwardsiella tarda*, *Morganella morganii*, *Proteus vulgaris*, and *Providencia stuartii* are divided into smaller groups by using via the H_2S test (TABLE 31). As can be seen from TABLE 31, all bacteria can be easily distinguished from each other by the ornithine decarboxylase and urease tests[11].

(B) Indole-negative bacteria

Indole-negative bacteria can be divided into smaller groups in order to distinguish among them by using the urease test (TABLE 32).

TABLE 32: Categorization of indole positive bacteria by using the urease test

Urease-positive bacteria	Urease-negative bacteria
Proteus mirabilis[1]	*Shigella sonnei*
Proteus penneri[2]	*Erwinia cacticida*
Yersinia pseudotuberculosis[3]	*Salmonella bongori*
	Serratia marcescens
	Salmonella enterica
	Salmonella enterica
	Serratia liquefaciens
	Yersinia pestis

[1]Ornithine decarboxylase test positive [2] Ornithine decarboxylase test negative, motility test positive [3] Ornithine decarboxylase test negative, motility test negative

Urease-negative bacteria can be divided into smaller categories by the motility test (see TABLE 33).

The purpose of the KCN test is to determine whether bacteria are able to grow in a medium in which potassium cyanide is present as a carbon and nitrogen source. To perform the KCN test, a small amount of pure culture of bacteria should be aseptically transferred to a sterile test tube of potassium cyanide (KCN) broth, which is a mixture of nutrient medium and 5% potassium cyanide. The above-mentioned tube should be incubated at 35-37 °C for 24 h. A positive result is indicated with the presence of turbidity in the tube[13,16].

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