

# Techniques for the identification of unknown microorganisms



**ASSIGN  
BUSTER**

## Identification of Unknown Microorganisms

For years and years, we have been trying to classify and understand microorganisms, and in doing so, we learned of certain techniques to identify them based on what they look like, where they live, and what they eat. We used this knowledge to derive simple tests to run, and we used a key to narrow down our choices, all in an effort to identify a microorganism.

Beginning in the earliest labs with the earliest microbiologists, the most common way to identify an organism is to look at it and look at what it does. In microbiology of bacteria, is it round (cocci) or rod-shaped (bacilli)? Does it stain purple (gram +) or red (gram -)? Does it use lactose as food, while creating an acidic product that can be observed? These tests based on the physiology/morphology and biochemistry fall under what is known as classic identification[1]. Classic techniques focus on the ability of a bacterium to grow and reproduce in certain conditions, such as the fact that bile salts can interact unfavorably with the peptidoglycan cell walls of gram positive bacteria, therefore selecting against their growth. Most tests are used to specifically select for or differentiate between the species, this in conjunction with a dichotomous key helps narrow down the choices until one is satisfactory. In contrast, modern methods of identification are based on the technological advances of recent years, namely, polymerase chain reaction (PCR) and genetics. The use of PCR to identify bacteria is rooted in the study of genetic coding, in particular, RNA. Every bacterium has strands of RNA that is used to bind the mRNA for translation: the 16s ribosomal RNA (rRNA) [2]. This sequence is conserved in all bacteria and can be used to identify species after amplification through the PCR process. Since every species has

a different genetic make-up, it is simply a matter of time for identification. But for most purposes, getting samples to a PCR and running them takes quite some effort, and a lot of money, thus the simpler and more efficient method is the classical testing.

At one point or another, we all have gotten the sore throat and cough. These symptoms are sometimes caused by microbes in the pharynx that shouldn't be there. Bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* can infect the area and cause this sore feeling. A more serious offender, *Streptococcus pyogenes*, causes what is commonly known as strep throat. These bacteria make the pharynx sore by harvesting erythrocytes and breaking them down as sources of food, in turn, destroying the lining and causing the all familiar sore feeling<sup>1</sup>. A simple test on a blood supplemented agar helps reveal what source of pathogen is causing the sickness. The agar is inoculated and incubated until growth is seen, and the plate will show a pattern of hemolysis if caused by one of the bacterial pathogens. Beta hemolysis is the ability to fully metabolize the cell and hemoglobin and will produce a clear halo around the colonies. Alpha hemolysis is the partial breakdowns of hemoglobin into biliverdin, making the halo appear yellow/green. Gamma hemolysis is the absence of halo, as it means the organism cannot lyse erythrocytes<sup>1</sup>.

## **Procedures:**

### **Respiratory swab:**

Take a sterile cotton swab and swab the tonsillary region

Inoculate blood agar by rolling the cotton tip in a streak plate method

After the initial line, discard the swab in biohazard bin, and continue with a flamed inoculation loop

**Second swab:**

Take a sterile cotton swab and swab the tonsilary region

Place the swab in a test tube

Dilute to 10<sup>-3</sup> and 10<sup>-4</sup>

Make a pour plate with nutrient agar

**Unknown identification:**

To start to identify any unknown organism by classical methods, it is necessary to create an appropriate dichotomous key to accompany the tests. The first step to any key is the gram stain; this essentially splits the number of possible organisms in half, gram positive and negative. Further tests based on morphology and biochemistry will select and differentiate between common characteristics of species until one organism stands out. At the start of every lab day, fresh streak plates and broths are prepared to keep the organism isolated and fresh.

Take a single colony and prepare streak plates, one for 37°C and one for 25°C

Prepare broths aseptically in similar fashion

**Dichotomous key:**

The dichotomous key was prepared using only the organisms on the list provided by Dr. Maxwell.

## Gram stain:

The gram stain separates bacteria into two main groups, gram positive and gram negative. The peptidoglycan cell walls of gram positive organisms will absorb the crystal violet stain and appear purple under microscope inspection. The gram negative bacteria lose their outer membrane during the decolorization step and will absorb the safranin stain, appearing red.

Prepare specimen by taking an isolated colony and heat fixing on a slide

Apply crystal violet stain for 60 sec

Wash with water

Apply iodine fixer for 45 sec

Wash with water

Rinse with decolorizer for exactly 8-10 sec

Wash with water

Apply safranin for 60 sec

If gram positive, the next step in my dichotomous key is to differentiate based on morphology: is it a cocci or bacilli? This step will split the organisms by shape, essentially cutting the number in half.

Using the gram stain slide, look at the shape of the specimen under 100x oil immersion

Bacilli are rod-shaped

cocci are round

If bacilli, run the catalase test. The catalase test will reveal if the organism has catalase, an enzyme used to breakdown harmful oxidizing agents such as hydrogen peroxide, which could be made as a result of metabolism. A drop of hydrogen peroxide is added to the specimen placed on a slide. A positive result will cause bubbles to form, indicating presence of catalase.

Put a colony on a slide

Add hydrogen peroxide

Observe bubbles/no bubbles

If catalase negative, as in no bubbles formed, the organism is *Lactobacillus acidophilus*. The bacteria are part of healthy normal flora and aids in digestion[3].

If catalase positive, the endospore stain must be used for further classification. The endospore is a tough spore formed by some bacteria during harsh environments. The spore will be dyed green in contrast to a cell dyed red.

Prepare a specimen by heat fixing on slide

Cover the slide with bibulous paper

Add methylene blue

Place slide on steamer over a Bunsen burner

Steam for 7-10 minutes, applying additional methylene blue to prevent the paper from drying out

Remove slide and paper

Cool for no more than 30 seconds and rinse

Counterstain with safranin for 1 minute

Rinse and blot dry

If endospore positive, the bacteria is *Bacillus subtilis*. The organism is found readily in the soil and can form a tough endospore when conditions become unfavorable. *Bacillus* is also a great model organism for gram positive studies[4].

If endospore negative, the organism is *Corynebacterium diphtheriae*. This bacterium is the cause of diphtheria, a respiratory infection.

If the organism observed is cocci, run the catalase test. The catalase test will reveal if the organism has catalase, an enzyme used to breakdown harmful oxidizing agents such as hydrogen peroxide, which could be made as a result of metabolism. A drop of hydrogen peroxide is added to the specimen placed on a slide. A positive result will cause bubbles to form, indicating presence of catalase.

Put a colony on a slide

Add hydrogen peroxide

Observe bubbles/no bubbles

If catalase negative, the organism is *Enterococcus faecalis*. It is commonly found in the digestive tract of humans and is considered part of the normal flora. It does not produce catalase.

If catalase positive, the use of mannitol salt agar is used to differentiate between the staphylococcus and micrococcus bacteria. Mannitol salt agar is used to select for staphylococcus. *Staphylococcus aureus* will produce a yellow halo.

Inoculate the MSA plate with a isolated colony

Incubate at 37°C for 48 hours

Observe growth

If a yellow colored halo around the colony is observed, it is either *S. aureus* white or *S. aureus* gold. The way to differentiate between the two is the color of the colony itself, which respectively is, white or golden (yellow). *S. aureus* is part of the normal flora and what is the usually cause of infections and pimples.

If the MSA plate shows no yellow halo, then it is either *Micrococcus roseus* or *Staphylococcus epidermis*. The way to differentiate between them is the color of the colony itself. *Micrococcus* will appear red in color due to a



pigment it secretes[5], while *S. epidermis* will be a white color. Both are normal skin flora.

If the organism is gram negative, the first test to run is the lactose supplement phenol red broth. The purpose of this test is to indicate whether or not the organism can use lactose as a food source. Fermenting lactose will produce an acidic waste and cause the phenol red to change color. A positive test will turn yellow.

Inoculate a tube with an isolated colony

Incubate at 37°C for 48 hours

Observe color change

If the organism cannot utilize lactose, then perhaps it can use glucose as a food source. The glucose phenol red broth tests for a similar metabolic process as the lactose one.

Inoculate a tube with an isolated colony

Incubate at 37°C for 48 hours

Observe color change

If the bacteria are glucose negative, then it is either *Pseudomonas* (*Burkholderia*) *cepacia* or *Pseudomonas aeruginosa*. The two can be differentiated by the pigment of the colonies. *P. cepacia* will appear a light purple color while *P. aeruginosa* will be white, and also produce a green pigment known as pyocyanin[6].

If glucose positive, a citrate slant must be prepared. The citrate slant tests for the ability to use citrate as a carbon source. A positive result will turn the green slant blue.

Inoculate the slant with an isolated colony

Incubate

Observe color

If citrate negative, the bacteria is *Proteus vulgaris*. It is a bacterium that normally inhabits the intestines of humans and in certain cases can cause infections, albeit in most cases of immunocompromised individuals.

If citrate positive, the organism is *Serratia marcescens*. It also produces a red pigment that can be used to identify it. The bacteria are found throughout environments and can be observed upon bathtubs that don't get washed often[7].

If the organism can ferment lactose, then the citrate test is prepared. The citrate slant tests for the ability to use citrate as a carbon source. A positive result will turn the green slant blue.

Inoculate the slant with an isolated colony

Incubate

Observe color

If citrate negative, the bacteria is *E. coli*. This is one of the most used model organisms for gram negative studies in labs. The rod shaped bacteria can cause some food poisoning if ingested. But the best use of it is in the labs, as it is easy to care for, replicates fast, and genetically simple, allowing for genetic research[8].

If citrate positive, the bacteria could be either *Enterococcus* or *Citrobacter*. The test to differentiate between the two is a Methyl red & Voges Proskauer media. The MR-VP is used to differentiate bacteria based on the ability to hydrolyze dextrose and create stable organic acids during fermentation. The MR-VP test combines the two separate tests. The positive result for the methyl red test is a red color when the methyl red indicator is added to the media. The positive result for the Voges Proskauer test is a red color.

Inoculate two MR-VPs

Incubate

Add the MR reagent to one tube and observe any color change every 10 mins

Look for any color change for the VP test before addition of reagent

If MR-VP positive, the organism is *Enterobacter*. *E. aerogenes* can cause infections and is considered pathogenic; however it still does exist as part of the normal flora of the human intestines.

If MR-VP negative, the organism is Citrobacter. They are ubiquitous in the environment and also inhabit the intestines.

## **Results:**

### **Respiratory swabs:**

The streak plate to identify organisms with hemolytic abilities showed growth of many different bacteria morphologies. Green and white colonies were observed to be growing on top of the plate. The media remained red throughout; no indication of any white spots. The pour plates for were both too numerous to count.

### **Unknown organisms:**

The organisms were isolated using a streak plate. Two isolations were obtained at two temperatures, 25o C and 37o C. the plate at room temperature showed red pigmented colonies (B), while the body temperature plate showed thick white colonies (A). A third unknown was given pre-inoculated (C).

Three biochemical tests and two morphological tests were used to identify the first unknown organism after isolation. A gram stain revealed that the organism was gram positive; being violet under the 100x oil immersion lens. Also while under the microscope, it was revealed that organism A was cocci. A catalase test was done and organism A was determined to be catalase positive, producing bubbles after addition of hydrogen peroxide. The unknown bacteria were inoculated on a mannitol salt agar, which showed the halo, a positive result. To identify between the two staphylococci aureus, the pigment of the colonies was used: white.

A gram stain revealed that organism B was gram negative; red under the 100x oil immersion lens. A lactose and glucose tube was inoculated in the same period to identify the ability to utilize those sugars. The organism was not able to metabolize lactose and was able to use glucose. A citrate slant was inoculated, after 48 hours the butt and slant of the citrate slant was turned blue; positive result.

The third unknown was tested to be gram positive. A catalase test came out negative, producing no bubbles.

## **Conclusions:**

### **Respiratory swab:**

Because no halo or clearing of any kind was observed in the media around the colonies, it can be safely said that none of the organisms in our throat swabs contained bacteria capable of hemolysis. This lack of hemolytic ability is known as gamma hemolysis. The pour plates were TNTC. This is probably because the dilutions performed were not enough; too many organisms were taken per tonsil swab. At the time, both participants were mildly sick and had seasonal allergies, possibly contributing to the amount of organisms per swab.

### **Unknown organisms:**

Three unknown organisms were presented for identification. Organism A was successfully identified as *Staphylococcus aureus* white. What led to that identification is that *S. aureus* is a gram positive cocci that produces catalase and is able to use mannitol and grow on the mannitol salt agar and produce the halo; it was determined that the color of the colonies was white as

opposed to gold. *S. aureus* is a common bacteria found on the skin and part of human normal flora. The bacteria can cause minor infections of the skin and can sometimes cause serious diseases and infection if it reaches the systemic circuit[i].

Organism B was identified as *Serratia marcescens*. It is a gram negative rod that can ferment glucose and citrate, but not lactose. It is a common environmental bacterium that can sometimes cause infections in skin and the urinary tract. *S. marcescens* is part of the family enterobacteriaceae and produces a red pigment that can be found on bathtubs and tiles, feeding on soap residues[ii].

Organism C was identified to be *Lactobacillus acidophilus*. It is a gram positive rod that does not produce any catalase for hydrogen peroxide breakdown. The bacteria are part of *Lactobacillus* which ferments sugars into lactic acids. This bacterium is used often for the production of many dairy products such as cheese and yogurt. *L. acidophilus* is part of normal human flora and can be found on the skin or gut. Sometimes it is used as a probiotic supplement. It also prevents *Candida* from overgrowing in the female vagina[iii]. All three organisms were identified successfully with the use of classic techniques.