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Section 101

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Unknown #5

Enterobactericeae, *Enterobacter cloacae*

Micrococcaceae, *Micrococcus luteus*

Abstract

The objective of an unknown report is to isolate and obtain the identity of an unknown organism through conducting various tests. The tests performed to obtain the identity of unknown organism #5 were lactose fermentation, starch hydrolysis, hydrogen sulfide production, and Simmons Citrate. The organism is positive for lactose fermentation, negative for starch hydrolysis, negative for hydrogen sulfide production, and positive for citrate permease from the Simmons Citrate test. Based on these results the identity of the unknown Gram-negative organism is Enterobactericeae *Enterobacter cloacae* (*E. cloacae*), however *Enterobacter aerogenes* (*E. aerogenes*) also shares these properties. The identity between the two cannot be distinguished with any tests available, therefore, it was confirmed by the Professor to be *E. cloacae* and not *E. aerogenes*. The identity of the Gram-positive organism was identified through conducting tests for catalase, lactose fermentation, aerotolerance, and morphology. The Gram-positive organism is catalase positive, negative for lactose fermentation, an obligate aerobe, and possesses a coccus morphology. Based on these results the Gram-positive organism is Micrococcaceae, *Micrococcus luteus* (*M. luteus*).

Introduction

Microbes were have thought to have existed for a long time, but, in 1675, Dutch cloth merchant Antonie Van Leeuwenhoek was the first to confirm the existence of microbes by developing a lens that allowed microbes to be examined (Parker N. and Lister P., 2016). In 1847, Ignaz Semmelweis, a Hungarian obstetrician, noticed a relationship between mortality rate of mothers who gave birth and who was in contact with them. When medical students or physicians tended to the mothers, there was a 10-20% mortality rate; however, with midwives, the mortality rate was only 1%. He proposed the difference in mortality rate occurred because students and physicians would perform autopsies, not wash their hands, and then immediately begin working on mothers. Midwives, on the other hand, did employ regular handwashing. Once Semmelweis suggested that the physicians start washing their hands with chlorinated lime water, the mortality rate dropped to 1% as well (Parker N. and Lister P., 2016). Although Semmelweis demonstrated that washing hands led to fewer infections, his ideas never spread due to his own stubbornness and lack of support for his claims.

In 1867, Joseph Lister, a British surgeon, continued the work of Semmelweis to reduce mortality from postsurgical infections. He examined the Semmelweis' and decided to use carbolic acid spray as a disinfectant. This implementation was very successful and greatly reduced postsurgical infections (Parker N. and Lister P., 2016). In 1909, German physician Paul Ehrlich, synthesized the first antibiotic, Salvarsan, by screening arsenic containing compounds against rabbits. Salvarsan was the 606^{th} compound attempted and was effective at targeting *Treponema pallidum* (Parker N. and Lister P., 2016). Although an accident, the most significant discovery, the first natural antibiotic, can be accredited to Alexander Fleming in 1928. Penicillin

is an antibiotic produced by *Penicillium notatum* and is an effective antibacterial against streptococci, meningococci, and *C. diphtheria* (Parker N. and Lister P., 2016).

The scientists mentioned above have greatly contributed to the field of microbiology and the development of the modern world. An argument could be made that penicillin is the greatest discovery of all time. It revolutionized medicine, completely changing life as people knew it. This led to an era where the health and well-being of people increased exponentially. People (on average) were able to live much longer than previous generations of the pre-antibiotic era. The study of microbiology allows for people to understand pathogens, how to prevent their spread, and how to treat infections that, in the past, would have been life threatening.

Identifying unknown organisms is important because, when the cause of an infection is unknown, determining the microbe responsible allows for accurate treatment. For example, Laura Albert is an immunocompromised woman who suffers from frequent urinary tract infections (UTIs). She is unable to take general antibiotics due to antibiotic resistance and complications related to other ailments. As a result, the specific bacteria responsible for the UTI must be identified each time so specific treatment can be administered. Without the ability to determine the identity of the microbe, she would not be able to control or treat the infections because the bacteria have become resistant to general antibiotics.

To identify an unknown organism, it must first be isolated. Isolation can be done through a variety of different methods like utilizing selective media. After the organism of interest has been isolated, biochemical tests can be performed to gather the identity such as testing for the ability to ferment carbohydrates or produce certain enzymes. Another method is to perform PCR (polymerase chain reaction) and obtain the genomic sequence of the organism, allowing for identification.

The purpose of this unknown report is to demonstrate how a microbe can be identified from a mixed culture. Determining the identity of the microbe requires knowledge on both isolating bacteria and conducting tests needed to ascertain the biochemical properties. This report demonstrates the processes and methodology behind identifying an unknown microbe.

Materials and Methods

Prior to working on an unknown organism, aseptic technique and safety must be practiced. This involves washing hands and wearing gloves, protective eye-wear and lab coats. It is also necessary to ensure the sterilization of inoculating loops via incineration between each use. Furthermore, every slide or plate should be labeled with a name, date, section number, and organism (or unknown organism number in this case). The goal of aseptic technique is to prevent contact between microbes and potential contaminates.

The unknown organism was obtained from mixed culture containing Gram-positive and Gram-negative bacteria. Because it was in a mixed culture, the bacteria had to be separated using selective media. Selective media isolates growth by inhibiting other biochemical factors. Phenylethyl Alcohol (PEA) and MacConkey select for Gram-positive and Gram-negative bacteria respectively. After inoculating a PEA plate and MacConkey plate with the mixed culture, the plates were incubated for 24 hours (unless otherwise specified incubation is at 37ºC).

A Gram-stain was performed on a colony from each plate to confirm that the media selected for Gram-negative bacteria on MacConkey and Gram-positive bacteria on PEA. To perform a Gram-stain, an isolated colony is smeared onto a slide with water and then heat fixed. Afterwards, a Gram-positive control and a Gram-negative control are streaked onto the slide. Next, crystal violet is poured over the slide and left for 1 minute, rinsing with water once the minute is up. Iodine is added using the same procedure as crystal violet. Following iodine, decolorizer is added until the runoff is clear. Finally, the slide is covered with safranin for one minute and then rinsed with water. If the organism is a purple color, then it is Gram-positive and if it is pink it is Gram-negative. After each organism has been confirmed to be Gram-positive or Gram-negative, the same colony used to perform the gram stain should be used to inoculate a

TSA broth. A broth medium is more suitable method for storage as opposed to using a plate. Now, since the organisms have been isolated, biochemical tests can be done to determine the enzymes present and, identify the organism.

The lactose test is a good initial test because the result will eliminate roughly 50% of the potential organisms, in this case. The Phenol Red broth test is used to test for Lactose fermentation. To conduct the test, a sterile loop is inoculated from the broth of the Gram-negative organism and is mixed into the Phenol Red broth media. The media is incubated for 24 hours, afterwards the results can be observed. If the media turns yellow, the organism is positive for lactose fermentation, and if it remains red or turns pink, it is negative for lactose fermentation. Furthermore, if any bubbles are in the Durham tube then gas production occurred.

To test for hydrogen sulfide production, a Hektoen Enteric (HE) plate can be inoculated. To inoculate a HE plate, using a sterile loop broth culture is obtained and streaked evenly across the plate. The plate is allowed to incubated for 24 hours. If there is growth on the plate, the organism is likely a Gram-negative Enterobacteriacaea species. If the color of the growth is orange, the organism is positive for lactose fermentation. If the growth is green, the organism is negative for lactose fermentation. The presence of a black precipitate indicates a positive result for hydrogen sulfide production.

The starch hydrolysis test indicates if an organism possesses the enzymes amylase or oligo-1,6-glucosidase and can utilize starch as a source of energy. To perform this test a sterile loop is used to obtain and to streak the broth culture onto the plate or slant. After the plate/slant has been inoculated, it should be incubated for 24 hours. After incubation, iodine is added to the plate or slant. If there is a clearing around the growth of the organism when the iodine is added, the

organism is positive for starch hydrolysis and possesses either amylase or oligo-1,6-glucosidase. If there is no clearing when iodine is added, the organism is negative for starch hydrolysis.

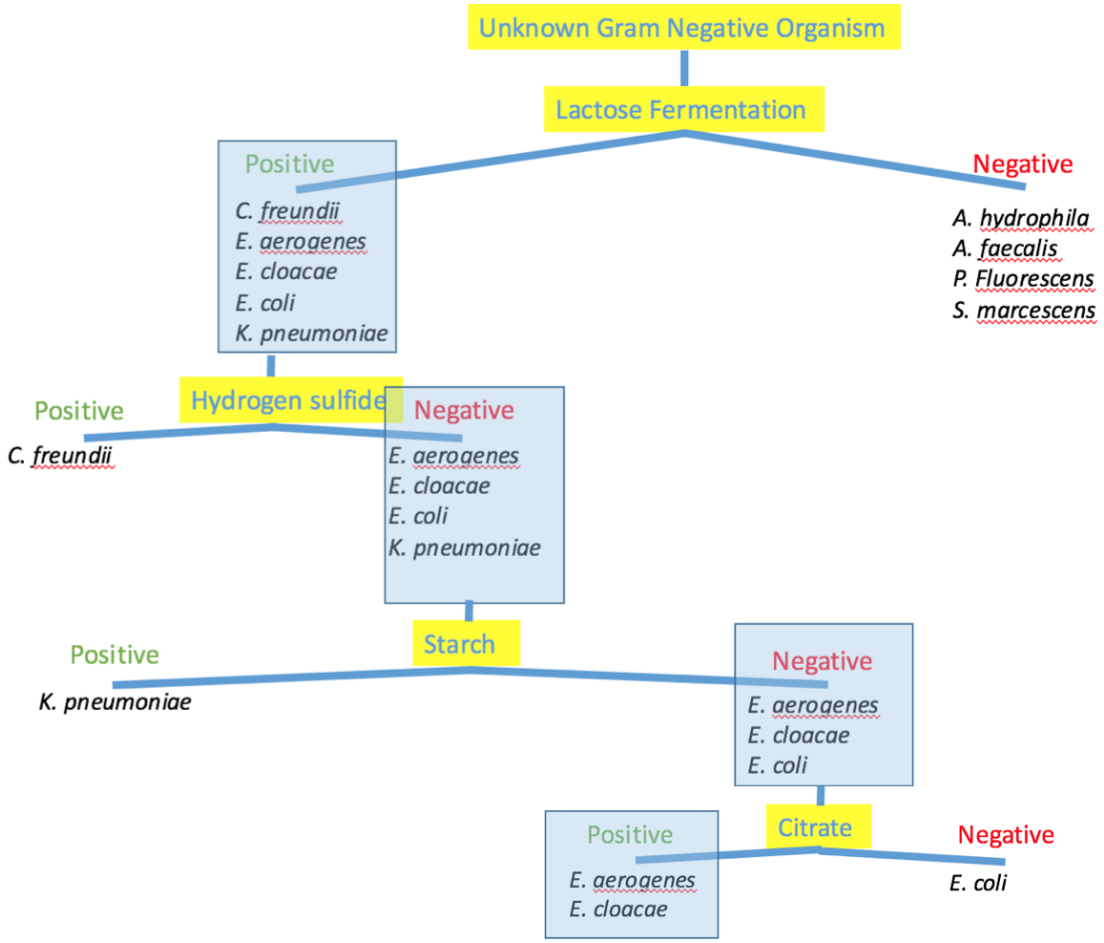
The Simmons Citrate Test is used to determine if an organism is able to produce the enzyme citrate permease and can utilize citrate as a source of energy. To perform this test a sterile needle is used to obtain broth culture of the organism. The media should be lightly inoculated with the needle so as not to put too much of the organism onto the media. The inoculated media should be incubated for 24 hours. If the media turns blue or there is growth without any color change, then the organism is positive for the presence of citrate permease. If there is no growth and no color change, the organism is negative for citrate permease.

Results

Test	Observation
Streak for Isolation (MAC plate)	Growth possessed slight pink hue
Gram Stain	Organism was pink after stain, as was the Gram-negative quality control
Lactose (Phenol Red Broth)	Red tube turned yellow and air was in the Durham tube
Hektoen Enteric (HE) Plate (Hydrogen sulfide Production)	Growth on the plate was green with slight orange coloration sparsely around the plate, no black precipitate present
Starch Hydrolysis	Slant possessed slight growth, when the iodine was added no clearing occurred around the growth
Simmons Citrate	The media changed from green to a blue color

Table 1. This table displays the observations of each test performed on the unknown Gram-negative organism

Flowchart



E. cloacae is the Unknown Gram Negative Organism

Figure 1. Flowchart depicting the tests performed to obtain the identity of the unknown Gram-negative organism. The blue box indicates whether the result of the test was positive or negative.

Discussion

Based on the results of the tests conducted, the identity of the organism was determined to be Enterobactericeae *Enterobacter cloacae*. The organism is positive for lactose fermentation, turning the tube from red to yellow. It is negative for hydrogen sulfide production, an absence of black precipitate on the plate indicates no sulfur reduction, negative for starch hydrolysis, no clearing occurred around the organism growth. Finally, the organism is positive for the Simmons Citrate test, the organism produces citrate permease and can utilize citrate to grow.

The Phenol Red broth test functions because the color of Phenol Red is pH dependent. When the pH of Phenol Red is less than 6.8 it turns a yellow color, it remains a red color when at a neutral pH (6.8 – 7.4), and turns pink when above 7.4. Lactose is then added to the broth with the Phenol Red, then the organism inoculates the broth and is incubated. If the organism is capable of fermenting lactose the pH of the broth will drop and the color will change from red to yellow. *E. cloacae* can ferment lactose which is why the tube turned yellow; as a result of the pH drop due to lactose fermentation.

Multiple tests could be conducted to test for hydrogen sulfide production, a Hektoen Enteric (HE) plate was chosen. The (HE) plate also tests for Lactose fermentation but was used primarily for the sulfur reduction test. Sodium thiosulfate is present in the media and can be utilized by the organism if it is capable of sulfur reduction. If sulfur reduction occurs, the hydrogen sulfide produced will react with ferric ammonium citrate in the media and a black precipitate will form. *E. cloacae* did not produce any black precipitate indicating it does not produce hydrogen sulfide.

The starch hydrolysis test determines if an organism produces amylase or oligo-1,6-glucosidase. If the organism produces either of those two enzymes, it is capable of hydrolyzing starch. To perform the test a plate with starch media is inoculated with an organism, after 24

hours of incubation, iodine is added. The iodine is an indicator, when it is added to the media it will form a complex with starch and turn a brown color. If there is not starch in the media, then no color change will be able to occur, indicating that the organism produces enzymes capable of hydrolyzing starch. *E. cloacae* did not have clearing when the iodine was added and turned a solid brown color indicating it does not possess amylase or oligo-1,6-glucosidase.

The final test performed was the Simmons Citrate test, which tests if an organism is capable of producing citrate permease and is capable of using citrate to grow. Sodium citrate is the only source of carbon in the media, requiring the citrate permease enzyme to bring citrate into the organism. Bromothymol blue is used in the media because it turns blue at a high pH and is green at low a pH. If the organism is capable of using citrate, the ammonium ion used as a nitrogen source will increase the pH, then the color of the media will turn from green to blue. A needle is necessary for inoculation because a loop will transfer too many organisms, allowing for recycling of carbon instead of utilizing citrate in the media. *E. cloacae* turned the media a blue color indicating it produces citrate permease and can utilize citrate as a carbon source.

The flow chart (Figure 1) shows how each result eliminated potential organisms and allowed for the identity of the unknown to be determined. The lactose test was important in eliminating about half of the potential organisms. Since the organism is positive for lactose fermentation, the pool of potential organisms shrunk significantly. Each test conducted afterwards was done to eliminate at least one potential organism. The final box in figure 1, below the citrate category, possesses two organisms, *E. aerogenes* and *E. cloacae*. The remaining tests to perform would yield identical results between the two organisms, so the professor had to confirm the identity of the unknown organism to be *E. cloacae*. The Gram-positive organism is catalase positive,

negative for lactose fermentation, an obligate aerobe, and possesses a coccus morphology. Based on these traits the Gram-positive organism is Micrococcaceae, *Micrococcus luteus* (*M. luteus*).

E. cloacae is an opportunistic pathogen that is often found in clinical settings like hospitals, causing nosocomial infections (Davin-Regli, A., & Pagès, J. M., 2015). It can be found in a variety of terrestrial or aquatic environments such as in food, soil, sewage, and water. *E. cloacae* spreads in hospital settings by contaminating equipment. It can cause infections like "bacteremia, endocartitis, septic arthritis, osteomyelitis, and skin/soft tissue infections…" (Davin-Regli, A., & Pagès, J. M., 2015). Some species even develop a resistance to third-generation cephalosporin's due to the production of the enzyme ß-lactamase (Modi, N and Cooke, R.W.I, 1987). In addition to resistance of third-generation cephalosporins in one case, *E. cloacae* is intrinsically resistant to first generation cephalosporin's, ampicillin, and amoxicillin (Davin-Regli, A., & Pagès, J. M., 2015).

Despite *E. cloacae*'s resistance to multiple ß-lactam antibiotics, fourth-generation cephalosporins and carbapenems are generally effective at treating an *E. cloacae* infection (there are some cases of resistance occurring even against these) (Fraser S.L., 2018). The spread of *E. cloacae* can be reduced in hospital settings by sterilizing and disinfecting surfaces or medical equipment; *E. cloacae* is prevalent by causing nosocomial infections due to improper sanitization.

M. luteus is generally found on the skin of humans as well as on "milk, goat cheese, and cassava fish." (Kundrat L., 2015). It is generally harmless but can be an opportunistic pathogen given the chance. It is associated with illnesses like "meningitis, septic arthritis, endocarditis, chronic cutaneous infections in HIV positive patients, and catheter infections." (Kundrat L.,

2015). However, it is not responsible for these infections it is just associated with them. It exists on human skin which is generally how it is transmitted to other people or the environment.

Some difficulties encountered in this Lab were that the Gram-positive bacteria, which was isolated and inoculated in a broth culture, was a mixed culture. The tests performed on what was thought to be a single Gram-positive organism were on two organisms which possessed similar traits .The only differing trait being morphology. After performing a streak for isolation of the broth it was determined that the broth culture was mixed.

Another difficulty was the starch media being a slant instead of on a plate, so when the iodine was added after the incubation process it was difficult to observe if clearing was occurring, or if iodine was simply dripping down over the growth from higher up “covering” the clearing. This issue occurred both times the test was performed. This made the results of the Starch test weak and unreliable, so it was not used to strongly eliminate potential organisms and more tests had to be performed.

The final problem encountered was the Gram-negative organism displaying a negative for the catalase test, despite all possible Gram-negative organisms being catalase positive. This same result held true even after confirming that the hydrogen peroxide was fresh when tested on a known catalase positive organism.

References

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