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Isolation and Characterization of Microbial Pathogens Found in Chickens

Kurt Lawton
Winona State University

John Weber
Winona State University

Appendix C

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project

Isolation and Characterization of Microbial Pathogens Found in Chickens

Student Name Kurt M. Lawton

Faculty Sponsor Dr. John S. Weber

Department Biology

Abstract

The bacterial content of retail chicken is a topic of growing concern. Chicken processing plants are under increasingly strict regulations to prevent contamination. Likewise, increasing controversy is being directed towards chicken farmers concerning cleanliness and the use of antibiotics in raising chickens, as it may lead to antibiotic resistance. There is legitimate concern surrounding the many possible pathogens associated with chicken, let alone the recent trend of bacteria with heightened resistance to antibiotics. This project was carried out with the purpose of the identification and characterization of bacteria found in chicken from local supermarkets. As multiple bacterial species were expected to be encountered, successful identification of at least one species was paramount for the continuation of this project prior to examining bacteria for antibiotic resistance and virulence factors.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, npeterson@winona.edu).

Student Signature Kurt Lawton Date 12/12/13

Faculty Sponsor Signature John S Weber Date 12/12/13

Isolation and Characterization of Microbial Pathogens Found in Chickens

Winona State University

Kurt Lawton and Dr. Jack Weber

December 3, 2013

Abstract
The bacterial content of retail chicken is a topic of growing concern. Chicken processing plants are under increasingly strict regulations to prevent contamination. Likewise, increasing controversy is being directed towards chicken farmers concerning cleanliness and the use of antibiotics in raising chickens, as it may lead to antibiotic resistance. There is legitimate concern surrounding the many possible pathogens associated with chicken, let alone the recent trend of bacteria with heightened resistance to antibiotics. This project was carried out with the purpose of the identification and characterization of bacteria found in chicken from local supermarkets. As multiple bacterial species were expected to be encountered, successful identification of at least one species was paramount for the continuation of this project prior to examining bacteria for antibiotic resistance and virulence factors.

Introduction
Chicken is available from a huge number of companies within the United States, some of which have been involved in controversies concerning contamination of their products with pathogenic bacteria. This experiment was designed to use a major brand chicken, since it was more likely to have a greater impact if potential bacterial pathogens were found. A Tyson brand chicken was nonspecifically chosen and purchased from WalMart of Winona, Minnesota to serve as the source of bacteria for analysis. No specific bacteria was targeted, but certain general categories were pursued including gram-positive staphylococci, streptococci, and enterococci and gram-negative enterics. This allowed for an open-ended experimental direction depending on the unique characteristics of the bacteria that arose. Multiple species of bacteria were expected to be found in each chicken, but for practicality in the short window of time for experimentation, only one species of bacteria was to be extensively analyzed. The most important factor dictating the direction of this experiment is a given specie's impact on humans. For this reason, the topics of greatest focus were to be microbes that could be resistant to antibiotics and may carry virulence factors. Being the first of Dr. John Weber's research students to pursue this topic, much ground work and technique development was needed. The experimental procedure for isolation of bacteria was carefully documented for the purpose of reproduction as new research students take over the reins and continue the research. With a well-documented procedure of an efficient way to isolate bacteria from chicken, future students will be able to spend less time developing procedures and more time testing for bacteria that carry antibiotic resistance genes and pathogenic virulence factors.

Materials and Methods
A mostly frozen Tyson chicken was acquired from WalMart. The chicken was thawed in a warm water bath for 30 minutes or less. The chicken was then unpackaged and opened to expose the inner cavity. 500 mL of nutrient broth was sprayed into to the inner cavity 30 mL at a time using a 30 mL sterile syringe, with a 16 gauge needle. The nutrient broth was shaken within chicken and pour**ed** into a 1 liter beaker. Ten sterile 30 mL centrifuge tubes were filled with nutrient broth. The broth was centrifuged at 12,000 RPM's for 10 minutes. After the supernatant broth was poured off, pellets from all 10 tubes were re-suspended in

a total of 10 mL of nutrient broth. The suspension served as source of bacteria for primary inoculations.

100 μl of the suspension was used to inoculate six flasks containing 50 mL of broth. Inoculation was two of each of the following broths; gram-negative (GN) broth, bile esculin (BE) broth, and m-staph broth. All flasks were incubated at 37 degrees Celsius for 48 hours in an agitator at 120 rmp. A sample of each of the six nutrient broths was frozen back at -80°C using 40% glycerol for a final concentration of 20% glycerol in the cryovial. Isolation streak-outs were preformed on 4 different agar plates: mannitol salts agar (MSA) for the m-staph, BE agar for the BE broth, and MacConkey's agar and EMB agar for the GN broth. Two of each type of agar plate were used for each applicable flask, for a total of 8 agar plates. Bacteria from the two GN broth cultures was used to inoculate MacConkey's and EMB agar plates. M-staph broth was used to inoculate MSA plates. BE broth was used to inoculate BE plates. The first attempted streak-out was unsuccessful at producing single colonies. Single colonies were isolated using a quadrant-streak method Eight agar plates were incubated at 37 degrees Celsius for 24 hours. Each set of matching agar plates were examined for individual colonies. One colony was chosen from each agar plate. The chosen colonies were picked, using a loop, to inoculate the same type of agar plate to obtain a pure culture. Eight agar plates were incubated at 37 degrees for 25 hours.

Gram stains were preformed on the eight pure culture samples for morphological identification. Colonies on MacConkey, BE, and MSA plates were used to inoculate 6 250mL of GN, BE and m-staph broths, respectively. This was to produce fresh cultures, still in growth phase, in a broth solution for a more successful freezing process and to ensure that pure cultures were stored in a frozen state. Broths were incubated at 37 degrees Celsius for 18 hours and then 37 degrees Celsius for another 12 hours in a shaker. 0.5 mL of each broth sample with added to a cryo tube, along with 0.5 mL of 40% glycerol. One cryo tube of each sample was frozen at -80 degrees Celsius. One colony found on the bile esculin agar had the ability to metabolize esculin, evident by hydrolysis of esculin to 6,7-dihydroxycoumarin, which reacts with iron to blacken the medium. This colony was chosen as the experimental bacteria, as enterococci have recently been associated with antibiotic resistance in poultry (7). A fresh growth of sample bacteria was streaked out from the bile esculin plate onto a new plate and incubated at 37 degrees Celsius,then used for initial differential testing. The test bacteria was streaked onto a blood agar plate to test for hemolysis and incubated for 24 hours at 37 degrees Celsius. The hemolysis test was chosen for it's ability to help establish which subsequent tests would be most appropriate. Using a PYR kit by Hardy Diagnostics the bacteria was smeared on a wetted-PYR disc on a slide. After two-five minutes, two drops PYR reagent were added, and the disc was examined 1 minute later. If the bacteria has the enzyme L-pyrrolidonyl arylamidase it will have the ability to hydrolyze the disk substrate L-pyrrolidonyl-ß-naphthylamide to create L-pyrrolidone and ß-naphthylamide. The reagent contains p-dimethylaminocinnamaldehyde which reacts with the ß-naphthylamide to form a red Schiff base, and therefore a bright pink or red positive test. Use of the PYR test in this project successfully narrowed the list of potential enterococci species possible as our unknown. 6.5% NaCl broth was inoculated with the esculin-positive unknown, to further rule out various species of enterococci or streptococci. Four flasks with 25 mL mannitol phenol red

broth and four flasks with 25 mL arabinose phenol red broth (0.5% L-arabinose) were autoclaved and inoculated to distinguish between species of enterococci. Broths were incubated at 37 degrees for 24 hours at 120 rpm.

Frozen samples of *E. faecalis* and *E. faecium* were used to inoculated one 25 mL portion of both mannitol and arabinose broth to be used as a control. PCR was to be used for confirmation of *E. faecalis*. Three 5mL portions of bile esculin broth was added to 3 separate test tubes, and in the same way three 5mL portions of nutrient broth. The suspected *E. faecalis*, the control *E. faecalis* and *E. faecium* were each used to inoculate one of each type of broth in the test tubes. The test tubes were inoculated at 37 degrees Celsius and 100 rpms for 24 hours. 2mL of each culture of bile esculin was added to 2mL screw-cap centrifuge tubes. Samples were centrifuged at 15,000g for 10 minutes. Supernatant was pour off of pellet. Pellet was resuspended in 100 µL of sterile water. Sealed tubes were placed in boiling water for 5 minutes and cooled on ice for 2 minutes. The PCR solution was set up with the following final concentrations per 25µl reaction; 1.5mM $MgCl_2$, 200µM dNTPs , 50µM or 50 pmoles of each primer, 1 unit (U) of Taq Polymerase, DNA and Water up to 25µl. Specifically for a 25µl the following amounts were used; 2.5µl of 10xBuffer, 0.75µl of 50mM $MgCl_2$, 1µl of 50µM stock of applicable forward primer, 1µl of 50µM stock of applicable reverse primer, 4µl of 1.25mM stock of dNTPs, 0.4µl of 5U/µl Taq Polymerase (Invitrogen), 2µl of DNA, sterile water to bring total volume to 25µl or, in this case, 13.35 µl. All reagents in PCR solution, except for the DNA, were mixed together and then added to PCR tubes to increase accuracy of liquid measurements. The primer sets used were; fcls-gdh-f GGCGCACTAAAAGATATGGT (*E. faecalis* forward), fcls-gdh-r CCAAGATTGGGCAACTTCGTCCCA (*E. faecalis* reverse), fcm-gdh-f GGCGCACTAAAAGATATGGT (*E. faecium* forward), fcm-gdh-r CCAAGATTGGGCAACTTCGTCCCA (*E. faecium* reverse)(4, 5, 11). PCR was carried out in a thermal cycler using the following conditions: 94^{o} C for 4 minutes, 30 cycles at 94^{o} C for 1 minute, 53 ^{o} C for 40 seconds and 72 ^{o} C for 40 seconds. After the final cycle, there was a final extension of 2 minutes at 72 ^{o} C. 10x gel-loading dye was added to the tubes and 6ml of PCR products were immediately inserted into wells of an electrophoresis gel and run along markers at 104V/38A for 50 minutes. The gel was then stained with ethidium bromide by submersion for 15 minutes. Destaining of the gel followed staining by 5 minutes of agitated submersion in TAE buffer solution. After staining the gel was viewed under ultraviolet light and photographed.

Antibiotic-Sensitivity Testing: Two 150mm Mueller-Hinton agars were inoculated with the unknown bacteria from a 17-hour BE broth culture. Antibiotic discs were applied to the inoculated agars at least 45mm from one another. Agar was incubated at 37 degrees Celsius for 17 hours. The plates were analyzed for antibiotic resistance by measuring the zones of inhibition and comparing them to standards (1 (pg. 432)).

Results, Conclusions and Discussion

Culture Development

In the initial inoculation, all media allowed for growth of bacteria. Likewise the inoculations of the differential media following the initial inoculations exhibited growth in all media. Most agars exhibited two visibly different colonies.

Agar	Bile Esculin	MacConkey's	EMB	M-Staph
Different colonies	2	2	2	2
Description	One colony metabolized esculin, other did not	One colony type was pink/purple, other colony type was clear	One colony had a green sheen, one was purple/gray	Both caused yellowing of agar, mannitol fermentation

Three different selective and enrichment broths were used for differential bacterial propagation: Gram-negative (GN) broth, bile esculin (BE) broth, and m-staph broth. These media were chosen for the purpose of selecting and encouraging the growth of bacteria, especially pertinent to the project focus. GN broth is selective towards gram-negative enteric pathogens. Bile esculin broth is selective towards enterococcus and some streptococci species. M-staph broth is selective toward staphylococci species.

For the isolation of gram-negative enteric pathogens, GN broth was inoculated with 100 µl nutrient broth from the chicken cavity. A sample of the inoculated GN broth was frozen back before it was used to inoculate two EMB agar plates and two MacConkey's agar plates. Single colonies were isolated from each pair of media. Two types of bacteria were isolated from MacConkey's agar, one a lactose fermenter and one that did not ferment lactose, both of which were frozen back. The same two types of bacteria were isolated from EMB agar, both of which were also frozen back.

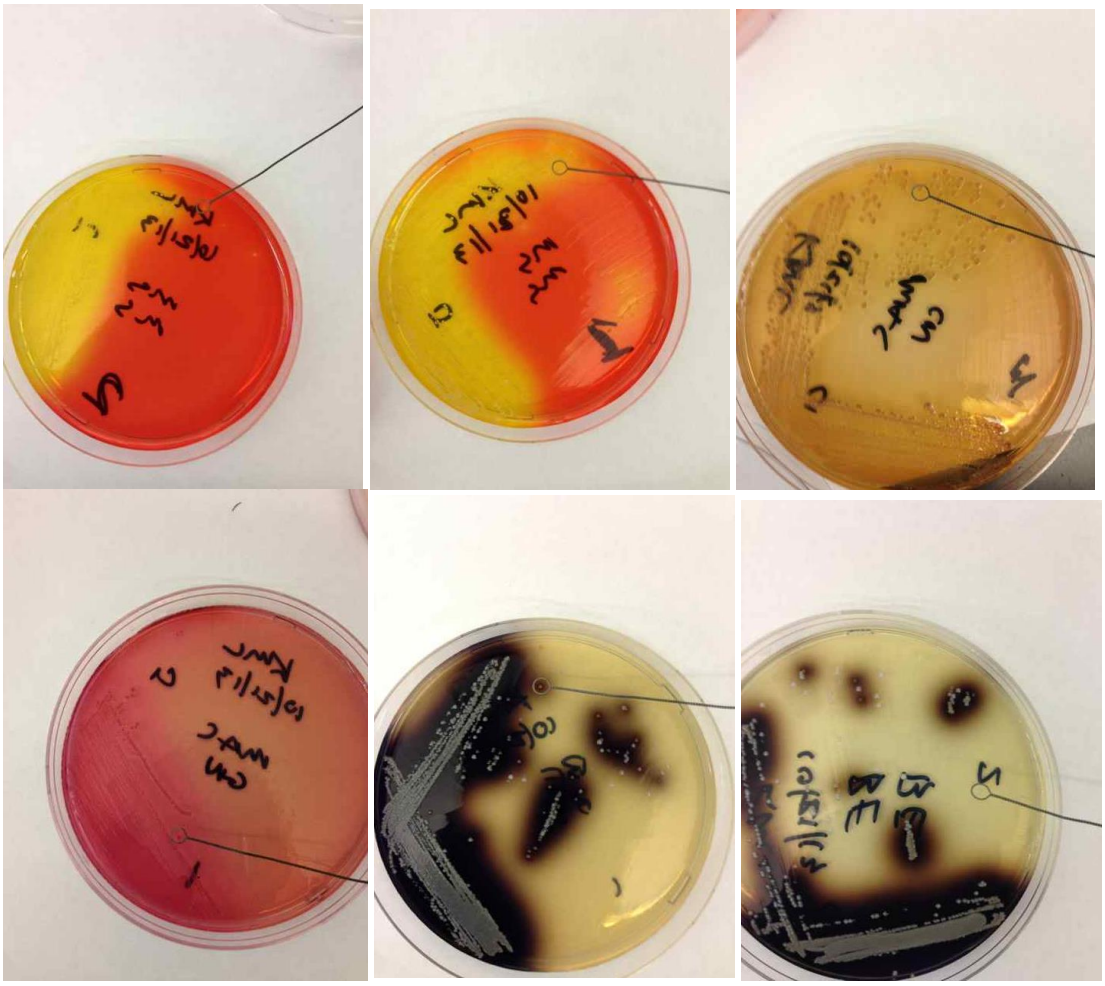
For the isolation of enterococci and group D streptococci, BE broth was inoculated with the nutrient broth rinsed with a chicken cavity. A sample of inoculated BE broth was frozen back and was then used to inoculated two bile esculin agar plates. Single colonies were isolated from each plate, one able and the other unable to metabolize esculin. A isolate of each bacteria was frozen back.

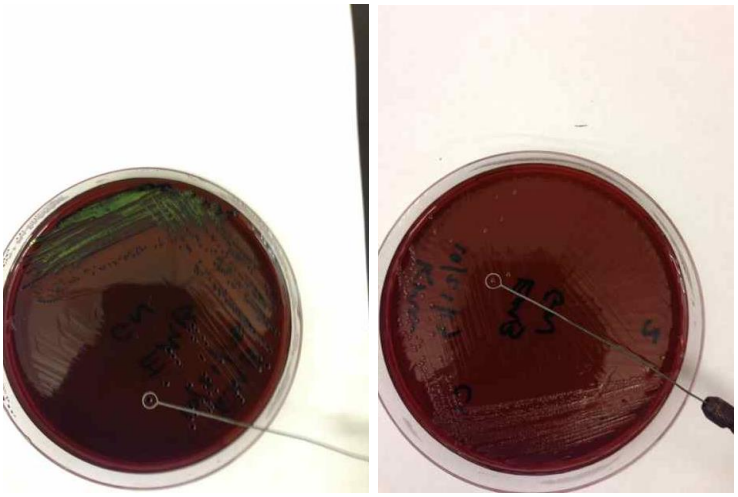
For the isolation of staphylococci, m-staph broth was inoculated with 100 µl nutrient broth used to rinse the chicken cavity. A sample of the inoculated m-staph broth was frozen back before it was used to inoculate two MSA plates. A single colony was isolated from the plates, capable of mannitol fermentation, and was frozen back.

Pure Culture Colony Selection

Two of each type of media were inoculated, one plate for each unique colony type from each type of media with eight plates total.

Agar	Bile Esculin	MacConkey's	EMB	M-Staph
Plate A	Esculin is metabolized	Pink agar	Green sheen	Yellowing by colonies
Plate B	Esculin not metabolized	Tan agar	Purple/gray colonies	Yellowing by colonies





Plates had pure colonies, but a second single colony streak out was carried out to ensure purity.

The results of the bile esculin plate allowed me to narrow down the possible species to enterococci or group D streptococci. I focused on the unknown that could metabolize esculin. This result suggested that it was a species of enterococcus, and some species of enterococcus are a major source of nosocomial infections, especially in surgical wounds. Therefore, it is important to determine if this unknown fell into that category. To distinguish between species in this category, a blood agar test, 6.5% salt broth test, and PYR assay were done. (See table in (2 (pg. 457); 9)

Blood Agar Test
Gamma hemolysis was visible on three consecutive inoculations of blood agar with the bile esculin hydrolysis-positive unknown bacteria.

Trial 1	Trial 2	Trial 3
Gamma	Gamma and Alpha	Gamma

High Salt Test
6.5% NaCl broth exhibited growth.

PYR Test
Unknown tested positive with PYR kit. *E. faecalis* and *E. faecium* samples used in this project were not tested; literature results were used in both cases. *E. faecalis* and *E. faecium* are both positive for the PYR test. (2)

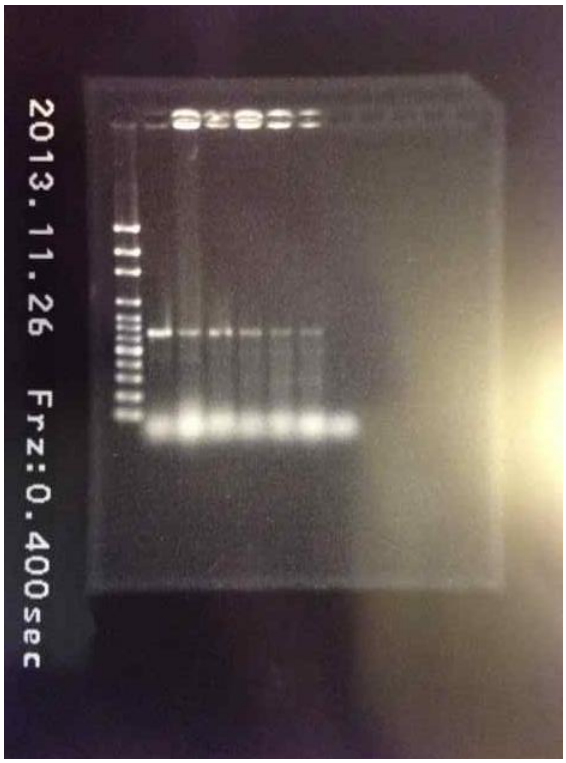
Mannitol and Arabinose Carbohydrate Test
E. faecalis and *E. faecium* were used as controls.

Bacteria	Mannitol	Arabinose
E. faecalis	positive	negative
E. faecium	positive	positive
Unknown	positive	negative

Arabinose left, mannitol right.

PCR Reactions

Gel 1



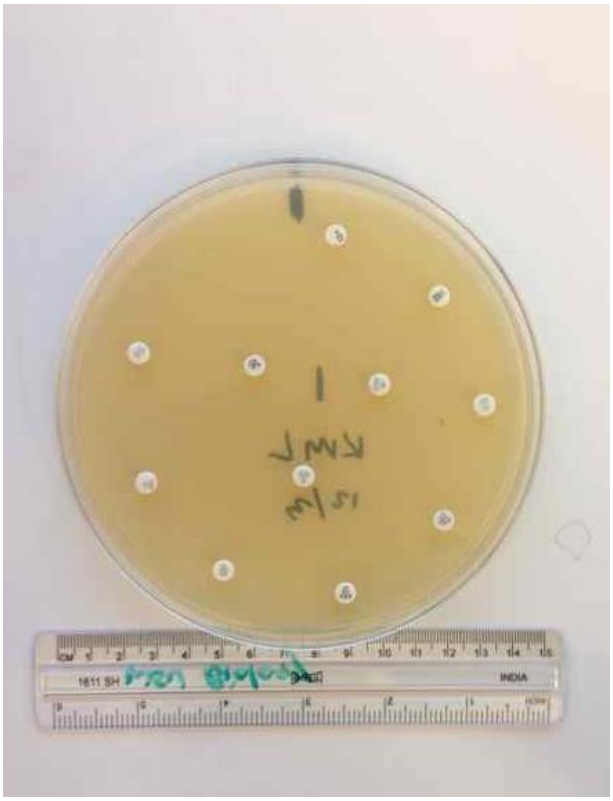
Lanes 1-8 (Left to right)
1: 100 bp Marker
2-3: *E. faecium* DNA, Efcm primers
4-5: *E. faecalis* DNA, Efcm primers
6-7: Unknown DNA, Efcm primers
8: No DNA, Efcm primers

Gel 2

Lanes 1- 8 (Left to right)
1: 100 bp Marker
2-3: *E. faecium* DNA, Efcls primers
4-5: *E. faecalis* DNA, Efcls primers
6-7: Unknown DNA, Efcls primers
8: No DNA, Efcls primers

Antibiotic Sensitivity Testing (1 (pg. 432)

Antibiotic	Zone of Inhibition	Resistence
Erythromyacin	19mm	Intermediate
Amikacin	None	Resistant
Chloramphenicol	28mm	Sensitive
Carbenicillin	26mm	Sensitive
Ampicillin	20mm	Sensitive
Nalidixic Acid	None	Resistant
Gentamicin	None	Resistant
Vancomyacin	17mm	Sensitive
Penicillin	10mm	Intermediate
Streptomyacin	18mm	Sensitive
Ceftriaxone	18mm	Intermediate
Ciprofloxacin	16mm	Intermediate
Streptomyacin	18mm	Sensitive
Cefazolin	17mm	Intermediate
Sulfisoxazole	None	Resistant
Oxacillin	None	Resistant
Optochin	None	Resistant
Tetracycline	None	Resistant
Kanamyacin	None	Resistant



Culture Development

No specific bacteria were anticipated in the primary inoculation. Some categories of bacteria were passively pursued by the use of gram-negative broth, bile esculin broth, and m-staph broth. By use of these media we would likely encounter gram-negative pathogenic bacteria, enterococci/streptococci, and staphylococci. These categories are important because many bacteria in these categories carry antibiotic resistance genes and virulence factors. Furthermore, by promoting the selection of these bacterial groups, it will take less time to identify the species within each group and lead to more rapid analysis of genes important in increasing virulence and causing resistance to antibiotics.

Pure Culture Colony Selection

Bacteria grew in every media that was inoculated, many of which exhibited multiple unique colonies. For practicality, no more than two different colonies were used from each agar for further isolation. It was originally assumed that there would not be so many different types of these categories of bacteria present in the chicken. The mere presence of so many potentially pathogenic bacteria is alarming and is grounds for further study, but was outside the scope of this project. Based on the initial differential and selective screen, seven unique bacterial colonies were isolated from the 8 differential plates. The esculin-positive colony was chosen for further study as it was likely to be an enterococcus, which is a genus of great interest, especially since many medically important enterococci are resistant to antibiotics. Enterococci have recently appeared in studies as a microbe prone to carrying an antibiotic gene. The relevance is even greater as enterococci are known to cause some of the most serious infections in humans. Establishment of enterococci within a human could lead to serious problems due to antibiotic resistance and virulence factors. (10)

Blood Agar Test

The blood agar test was the first test preformed on the unknown bile esculin-positive bacteria. A blood agar test was not highly differential within the enterococcus genus, but it was helpful in ruling out some unlikely possibilities.

PYR Test

The PYR test was positive, which supported our suspicion of *E. faecalis or E. faecium*. (2)

Mannitol and Arabinose Carbohydrate Test

E. faecalis and *E. faecium* are both common isolates found in poultry leading us to strongly suspect either as the unknown. To differentiate between the two, a carbohydrate test was performed using arabinose and mannitol. While both species are capable of metabolizing mannitol, only *E. faecium* can metabolize arabinose. *E. faecalis* and *E. faecium* were used as a control. The unknown was able to metabolize mannitol but not arabinose, leading us to suspect it to be *E. faecalis.* All the data support the idea that the unknown isolated from the chicken was *E. faecalis*.

PCR

The most accurate way to identify a species of bacteria is through PCR. Since the unknown was very likely to be *E. faecalis*, it would be the final step of identification to run PCR.(6). The results of the PCR reaction were unclean and indecisive. This may have been due to improper primers or a need for adjustment in the solutions used to run PCR, more than likely the latter.

Antibiotic Sensitivity Assay

Antibiotic resistance in pathogens is one of the biggest concerns in global health currently. Inability to use antibiotics opens the door to devastating plagues like those seen in days past. A whole semester could be used for the study of *E. faecalis* and its potential antibiotic resistance. Finding *E. faecalis* is significant because it has been identified to have antibiotic resistance genes that could be medically threatening (8). Isolates of *E. faecalis* have been found with resistance to all medically administered antibiotics. Furthermore, *E. faecalis* is often associated with surgical infections and compound medical complications. Even more relevant to this project, *E. faecalis* populations are thought to be increasing in number within chicken as a result of antibiotic use in chicken feed. The culture development and preliminary set up of this research took the majority of the semester, leaving very little time to study the antibiotics. From the first round of Kirby-Bauer testing, all antibiotics available at Winona State University were used. Some of these antibiotics were not relevant to *E. faecalis* based on the literature, but were used anyway in case our strain of *E. faecalis* was unique. Reaction to most of the antibiotics was not unexpected, but it is too early to come to conclusions in this area. As this research expands and other students take the work further, testing with oxytetramycin will be important. Currently there is controversy over the use of oxytetramycin in raising poulty. Some researchers claim to have evidence of antibiotic resistance developing in poulty, chiefly chickens and turkeys, as a result of use of this antibiotic. There is currently no restriction on the use of this antibiotic in chicken feed, many are fighting for the imposition of regulations to fight abuse which may lead to antibiotic resistance.

This research topic coincides with many pertinent topics in health and microbiology. The pathogens found in poultry continue to pose a risk to human health. Understanding and discovering virulent and antibiotic resistant bacteria will help us in efforts to protect ourselves and help to understand causes of antibiotic resistance. The techniques described in this paper provide a framework for identifying bacteria found in chicken. The groundwork for isolating a pathogen for further testing for antibiotic resistance and virulence factors is in place. This will allow subsequent research students to focus more quickly and efficiently on characterization of bacteria rather than on isolation and preliminary identification. Furthermore, there are five or more other species bacteria that have been isolated during this project. These bacteria need to be identified and characterized for a full analysis of the potential pathogenic bacteria carried by store-bought chickens. There is much to be studied in the fields of virulent and antibiotic-resistant bacteria that is within the scope of Winona State University research students. Now that proper technique has been developed in isolating the pathogens, more time can be spent discovering the critical characteristics of these pathogens.

Bibliography

1. Bergey, D. H., and John G. Holt. *Bergey's manual of determinative bacteriology*. 9th ed. Baltimore: Williams & Wilkins, 1994. Print.

2. Brown, Alfred E., and Harold J. Benson. *Benson's microbiological applications: laboratory manual in general microbiology, complete version*. 12th ed. New York: McGraw-Hill Higher Education ;, 2010. Print.

3. "Enterococcus Information and Courses from MediaLab, Inc.." *Enterococcus Information and Courses from MediaLab, Inc.*. N.p., n.d. Web. 15 Dec. 2013. <http://www.medialabinc.net/enterococcus-keyword.aspx>.

4. "Enterococcus faecalis - Organismal Information." *Enterococcus faecalis - Organismal Information*. N.p., n.d. Web. 12 Dec. 2013. <http://efaecalis.mlst.net/misc/info.asp>.

5. "Enterococcus faecium - Organismal Information." *Enterococcus faecium - Organismal Information*. N.p., n.d. Web. 15 Dec. 2013. <http://efaecium.mlst.net/misc/info.asp>.

6. Franzetti, Laura, Mariagrazia Pompei, Mauro Scarpellini, and Antonietta Galli. "Phenotypic And Genotypic Characterization Of Enterococcus Spp. Of Different Origins." *Current Microbiology* 49.4 (2004): 255-260.

7. Hayes, J. R., L. L. English, P. J. Carter, T. Proescholdt, K. Y. Lee, D. D. Wagner, and D. G. White. "Prevalence And Antimicrobial Resistance Of Enterococcus Species Isolated From Retail Meats." *Applied and Environmental Microbiology* 69.12 (2003): 7153-7160.

8. Larsen, Jesper. "Porcine-Origin Gentamicin-Resistant Enterococcus Faecalis In Humans, Denmark." *Emerging Infectious Diseases* 16 (2010): 682-684.

9. Manero, A., and A.R. Blanch. "Identification of Enterococcus spp. with a biochemical key.." *Appl Environ Microbiol* 65 (1999): n. pag. *PubMed*. Web. 15 Oct. 2013.

10. Rathnayake, Irani, Megan Hargreaves, and Flavia Huygens. "SNP Diversity Of Enterococcus Faecalis And Enterococcus Faecium In A South East Queensland Waterway, Australia, And Associated Antibiotic Resistance Gene Profiles." *BMC Microbiology* 11.1 (2011): 201.

11. Sedgley, C. M., A. Molander, S. E. Flannagan, A. C. Nagel, O. K. Appelbe, D. B. Clewell, and G. Dahlen. "Virulence, Phenotype And Genotype Characteristics Of Endodontic Enterococcus Spp.." *Oral Microbiology and Immunology* 20.1 (2005): 10-19.