RESEARCH REPORT
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Title: Culture-independent analysis of microbial communities in tonsils of healthy, carrier, and diseased pigs - **NPB #09-072**

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Industry Summary:

Many bacterial pathogens of pigs can be found in the tonsils, and the tonsils can act as a reservoir for these pathogens, allowing them to persist in a herd. The goal of this study was to characterize the bacterial community, or microbiome, in the tonsils of healthy pigs and further to compare the tonsillar community in healthy pigs to that in pigs with known infectious diseases. We used a combination of bacterial culture, the traditional method, and current culture-independent techniques, which allowed us to identify many difficult-to-culture bacteria. This study provides the first detailed characterization of the bacterial community, or microbiome, found in porcine tonsils. We identified bacteria found in the tonsils of normal, healthy pigs, and found a diverse mixture of aerobic and anaerobic bacteria that were primarily non-pathogenic commensal organisms. We then compared these to bacteria found in pigs with known infectious diseases. In many herds with disease, we saw increased numbers of the bacterial pathogens in the tonsils as well as a shift in the remainder of the bacterial community. For example, in a herd with chronic *Streptococcus suis* problems, there were significantly more *Streptococcus* in the tonsils than we found in healthy herds, as well as reduced numbers of several of the types of commensal bacteria. It is not yet clear whether a shift in the community allows the pathogen to increase or acquisition of the pathogen shifts the rest of the community; it will take additional experimental infection studies to answer this question. We also observed an effect of antibiotics on the normal bacterial community. In herds treated with antibiotics, we frequently saw increased numbers of anaerobic organisms in the tonsils, and a shift in the overall community. Whether this shift affects the subsequent acquisition of pathogens or development of disease is not known, although a parallel would be the effect of antibiotics on human intestinal bacteria.

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Scientific Abstract:

Porcine tonsils are the colonization site for many pathogenic as well as commensal microorganisms and are the primary lymphoid tissue encountered by organisms entering through the mouth or nares. Pathogens such as *Actinobacillus pleuropneumoniae, A. suis, Haemophilus parasuis, Pasteurella multocida, Mycoplasma hyopneumoniae*, and *Streptococcus suis*, some of the most important causes of bacterial pneumonia, septicemia, polyarthritis, and meningitis, are carried asymptomatically in the tonsils and nasopharynx of pigs. While vaccines that protect against serious disease caused by some of these pathogens have been developed, these vaccines generally do not prevent acquisition of the organism and carriage in the upper respiratory tract. Carriage can result not only in perpetuating the reservoir of the pathogen, but also in reduced growth efficiency, which can in turn lead to the use of antibiotics in feed as growth promoters. To further reduce or eliminate disease caused by these pathogens, and to reduce the use of antibiotics as growth promoters, there is a critical need to understand how the carrier state is established and maintained and to determine effective management strategies to reduce carriage of pathogens in the upper respiratory tract.

The objective of this project was to analyze the microbial communities in tonsils from healthy pigs and compare these to the communities found in animals from herds experiencing disease problems due to specific pathogens. To accomplish this, we first characterized the composition and structure of the microbial communities in the tonsils of healthy pigs. Whole tonsils were collected at necropsy from twelve 16-week-old finisher pigs from two healthy herds. Tonsil brushes designed for this project were also used to collect samples from four of these animals. Bacterial DNA was isolated from each sample, amplified by PCR with universal primers specific for the bacterial 16S rRNA genes, and the PCR products sequenced using high throughput bar-coded 454-FLX pyrosequencing. An average of 13,000 sequences were generated from each sample. Microbial community members were identified by sequence comparison to known bacterial 16S rRNA gene sequences.

The microbiomes of these healthy herds showed very strong similarities in the major components as well as distinct differences in minor components. *Pasteurellaceae* dominated the tonsillar microbiome in all animals, comprising 60% of the total, although the relative proportions of the genera *Actinobacillus, Haemophilus,* and *Pasteurella* varied between the herds. Also found in all animals were the genera *Alkanindiges, Peptostreptococcus, Veillonella, Streptococcus* and *Fusobacterium*, as well as *Enterobacteriaceae* and *Neisseriaceae*, which comprise the "core microbiome" of porcine tonsils. Tonsil brushes yielded similar results to tissue specimens, validating the use of this non-invasive technique for future studies.

To compare the microbial communities found in healthy pigs to those found in pigs that are carriers of specific porcine pathogens, we collected tonsil brush specimens from 164 pigs from 12 herds, including two high health status herds and 10 herds with acute or chronic problems with *Actinobacillus pleuropneumoniae, Streptococcus suis, Salmonella, Haemophilus parasuis,* or mixed pathogens. Community bacterial DNA was isolated from 41 samples and controls and the 16S rRNA genes amplified and sequenced using Titanium pyrosequencing. An average of 2500 reads per sample and 15,000 reads per herd were generated and analyzed. Our working hypothesis, that the composition of the communities in the tonsils would differ between healthy and infected herds, was supported by the results. For example, there were increased numbers of *Streptococcus* in the tonsils of the pigs from a herd with chronic problems with *Streptococcus suis* (more than double the usual amount in healthy pigs) and increased *Enterobacteriaceae* in the tonsils of pigs known to shed *Salmonella*, but not in the tonsils of non-shedders from the same herd. However, in some herds, use of antibiotics also altered the tonsil microbiota, and this could not always be separated from effects of carriage of a pathogen. Further studies examining the effect of antibiotics on the tonsillar communities in healthy pigs, and on subsequent susceptibility to colonization by pathogens and development of disease, would likely clarify these results.

Introduction:

Many bacterial species that are pathogenic for humans and animals have their natural host as their only known reservoir. These pathogens are frequently found in a carrier state, that is, in an asymptomatic colonizing state, in a significant percent of the population, rather than actively causing disease. How these carried pathogens establish themselves as a part of the normal flora without causing disease, how they are maintained in this carriage state, and what occurs to trigger development of disease in carriers and transmission to new hosts are major areas of research interest in both human and veterinary medicine. Understanding these

processes could lead to new tools to block acquisition of these organisms, eliminating the reservoir, or to block the transition from carrier state to virulent state, eliminating development of systemic disease. To address this question, a first step is to determine the composition of the microbial community of the relevant site in healthy animals, which can then be compared to the community in carriers and diseased animals and also establish a baseline to follow changes in the microbial communities during the development of disease.

The reservoir for some of the most virulent bacterial pathogens of pigs is the upper respiratory tract, particularly the tonsils. Pathogens such as *Actinobacillus pleuropneumoniae, A. suis, Haemophilus parasuis, Pasteurella multocida, Mycoplasma hyopneumoniae*, and *Streptococcus suis*, some of the most important causes of bacterial pneumonia, septicemia, polyarthritis, and meningitis, as well as many enteric pathogens such as *Salmonella,* are carried asymptomatically in the tonsils and nasopharynx of pigs. While vaccines that protect against serious disease caused by some of these pathogens have been developed, these vaccines generally do not prevent acquisition of the organism and carriage in the upper respiratory tract. Carriage can result not only in perpetuating the reservoir of the pathogen, but also in reduced growth efficiency, which can in turn lead to the use of antibiotics in feed as growth promoters. To further reduce or eliminate disease caused by these pathogens, and to reduce the use of antibiotics as growth promoters, there is a critical need to understand how the carrier state is established and maintained and to determine effective management strategies to reduce carriage of pathogens in the upper respiratory tract. To begin to address this question, it was first necessary to characterize the normal tonsillar microbial community.

The objectives of this project were first to analyze the microbial communities in tonsils from healthy pigs and then to compare these to the communities found in animals from herds experiencing disease problems due to specific pathogens. Our working hypothesis was that the composition of the communities in the tonsils would differ between healthy and infected herds. We chose to use culture-independent methods, specifically sequencing of the conserved 16S rRNA genes, which can identify organisms present in a sample regardless of their specific growth requirements.

This research has produced the first in depth culture-independent characterization of the microbial community in the tonsils of pathogen-free pigs of the level of community heterogeneity from pig-to-pig and herd-to-herd [Lowe et al, 2011; Lowe et al, 2012]. Further, we have applied these techniques to analyze microbial communities in pigs from herds with disease problems, and have begun description of the differences between communities found in healthy pigs, sick pigs, and pigs that are asymptomatically carriers of pathogens. We have also identified differences in the tonsillar communities in animals receiving antibiotics. In the long term, continuation of this research may identify organisms with the potential for development as probiotics to inhibit colonization with specific pathogens and reduce disease incidence without the use of antibiotics. This research begins to provide a better understanding of the interactions between normal microbiota and pathogens within the porcine upper respiratory tract that allow establishment and maintenance of carriage of pathogens and development of systemic disease.

Objectives:

The specific objectives of this project were:

1. To determine the composition and structure of the normal microbial community of the tonsils of healthy pigs.
    a. Isolate bacterial DNA from tonsils of healthy pigs and use PCR amplification and 454 pyrosequence analysis of 16s rRNA genes to identify the bacterial species found in tonsils.
    b. Evaluate pig-to-pig variation within herds and herd-to-herd variation of healthy pigs.
    c. Compare two methods for collection of specimens: removal of complete tonsils at necropsy or use of tonsillar brushes on live pigs.

2. To compare the microbial communities found in healthy pigs to those found in pigs that are carriers of specific porcine pathogens.
    a. Utilize the same techniques to identify bacterial species found in the tonsils of pigs from herds with disease problems with known bacterial pathogens.
    b. Evaluate pig-to-pig variation within herds and herd-to-herd variation of carrier/diseased pigs.
    c. Compare the microbiota in tonsils of healthy and carrier/diseased pigs to identify species that differ between these groups.

Materials & Methods:

Animals:

Objective 1: Eight 18-20 week old pigs from a high health status herd with no recent history of respiratory disease (Herd 1) and four similar pigs from a currently healthy herd with a history of chronic but undefined respiratory problems (Herd 2) were randomly selected for use in this study. Both herds are farrow-to-finish operations weaning at 21 to 24 days of age, with similar management, located in mid-Michigan. Groups of similarly aged pigs were moved from the nursery to the grow-finish rooms all in-all out, although there was a common airspace via either connecting corridor (Herd 1) or connecting doors (Herd 2). Herd 1 Time 1 contains four Hampshire-Yorkshire crossbred pigs (pigs A-D) that were sampled in June 2007. These four pigs received no vaccinations or in-feed antibiotics. Herd 1 Time 2 contains four purebred Yorkshire pigs (pigs J-M) that were sampled two years later (April 2009). These pigs received Tylan (Elanco Animal Health, Indianapolis, IN) in-feed and were vaccinated against PCV2. There were no other significant differences in feed, vaccination, or medication between the two sampling periods for Herd 1. Herd 2 contains four Hampshire-Cambrough crossbred pigs (pigs E-H) that were sampled only once, in July 2007. This herd was also vaccinated against PCV2 and received Tylan in-feed. Additionally, Herd 2 received Pulmotil (Elanco Animal Health) until 8 weeks of age. The standard feed ration for Herd 2 was similar to that for Herd 1.

All pigs were taken off feed at least 3 hours prior to collection of specimens. Pigs were anesthetized by intramuscular injection of Telazole (6.6 mg/kg) and Xylazine (3.3 mg/kg) prior to transport from the farms to the necropsy facilities at the Michigan State University Diagnostic Center for Population and Animal Health. Pigs were euthanized within 30 minutes by overdose with a pentobarbital solution (Fatal-Plus, 100 mg/kg, Vortech Pharmaceutical, Dearborn, MI) delivered intravenously into the vena cava, following standard procedures.

Lung specimens from Herd 1 Time 1 pigs A-D and Herd 2 pigs E-H were aseptically sampled and cultured on blood agar and brain heart infusion agar containing 10 µg/ml NAD. No bacterial isolates were recovered from Herd 1 pigs. *Arcanobacterium pyogenes* was recovered from well-defined lesions in the lungs of 1 pig from Herd 2 (pig F).

Objective 2: Tonsil brush specimens (see below) were collected from swine herds in Michigan, Iowa, North Carolina, and Ontario, Canada. Two university research herds were chosen because they had no history of respiratory or systemic disease, were known to be free of *Actinobacillus pleuropneumoniae, Mycoplasma,* and PRRS, and were maintained under strict biosecurity. Additional herds were chosen because they had a documented history of infection with known pathogens; these included 3 herds with *A. pleuropneumoniae,* 2 with *Haemophilus parasuis,* 2 with *Streptococcus suis*, 1 with *Salmonella*, and 1 with an undiagnosed outbreak of respiratory disease. A farm questionnaire was completed by the veterinarian who identified and sampled each herd, which recorded the size and management style of the farm, vaccines given, antibiotic usage, infectious disease history, and diagnostic tests performed. In general, 6 specimens were collected from each farm, although where possible additional samples were collected from asymptomatic animals as controls. Overall, we collected 164 tonsil swab specimens, with the assistance of Dr. Roy Kirkwood, Michigan State University; Dr. Alejandro Ramirez, Iowa State University; Dr. Julie Funk, Michigan State University; Dr. James Kober, Swine Veterinary Services of Michigan, and Dr. Brad Thacker, Intervet.

Collection of tonsil tissue. For Objective 1, the entire right and left palatine tonsils were collected from 12 pigs at necropsy. Tonsils were placed into sterile glass Petri dishes where extraneous connective tissue was removed and tonsils were divided into four quarters using a sterile scalpel. One quarter of the right tonsil was combined with one quarter of the left tonsil and this combined sample was minced, placed into a sterile 15 ml conical tube, and immediately frozen at -20ºC for subsequent extraction of community DNA for analysis.

Tonsil brush design and use: To allow collection from a broader range and larger number of animals without the need for euthanasia and necropsy, we designed a brush for swabbing porcine palatine tonsils. The tonsil brush consisted of three parts: a 0.5" diameter soft-bristle test tube brush with approximately 5" of metal handle, an 8" long by 0.5" diameter wooden dowel used as a handle, and a guard for the brush made from 2 15 ml screw capped centrifuge tubes. The brush portion was prepared by cutting the brush head to a length of 1" and sealing the cut end with a drop of superglue to protect the pig and tissue from injury. Once dry, the brush was soaked for 1 hour in 10% hydrochloric acid to destroy any contaminating DNA, rinsed thoroughly with sterile H_2O, and allowed to dry completely. The guard was made by cutting the conical ends off two 15ml centrifuge tubes, taping the cut ends of the tubes together, and removing one of the caps. The brush was then

assembled by inserting the handle of the test tube brush into the end of the dowel and placing the guard over the brush. The guard was secured to the handle with a piece of autoclave tape. Assembled brushes were autoclaved in instant sealing sterilization pouches.

For swabbing porcine tonsils, the cap was removed from the guard and the brush (with the guard still in place) was inserted into the pig's mouth near the tonsil. The guard was then pulled back to expose the brush. Both the right and left tonsils were brushed for approximately 10 seconds each, with sufficient pressure to allow penetration of tonsillar crypts with the soft brush bristles, and at the same time with care not to cause tissue damage and bleeding. The guard was replaced and brush removed from the pig's mouth. The guard was discarded and the brush removed from the handle and placed into a 50 ml sterile test tube containing 20 ml of ice-cold 80% ethanol. Brushes were then stored at -20°C for subsequent extraction of community DNA. This procedure took less than a minute to perform. Animals were immobilized with a snare, and in some cases a speculum was inserted to hold the mouth open, but no anesthesia was required.

Isolation of community DNA.

DNA from the microbial communities in pig tonsils was extracted from tonsil tissues and from bacteria collected from tonsil brushes using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The concentration of community DNA was determined spectrophotometrically using a Nanodrop (Thermo Scientific, Wilmington, DE). Fifty percent of the yield was immediately archived at -80°C; the remaining DNA was used for polymerase chain reaction (PCR) amplification of the 16S rRNA gene.

Sequencing of 16S rRNA gene amplicons.

Three methods were used to sequence 16S rRNA amplicons from microbial communities in porcine tonsils:

1. In our initial study, we amplified the entire 16S rRNA gene with universal forward and reverse PCR primers (27F or 63F and 1389R) and constructed clone libraries of these amplicons [Lowe et al, 2011]. The clones were sequenced with the same 27F or 63F forward primers, generating ~400 bp of sequence covering the V1 & V2 (variable regions 1 & 2) regions of the 16S rRNA gene (see Fig. 1 below). In this study, 832 clones were sequenced, from the tonsils of 8 pigs from two healthy herds.

2. For the studies in Objective 1, we used 454 FLX pyrosequencing. Community template DNAs were amplified with primers designed by the Ribosomal Database Project (RDP) at Michigan State University [Cole at el, 2005]. These primers amplify ~200 bp of sequence covering the V4 region of the 16S rRNA gene. The PCR products from 20-40 samples were combined in equal mass amounts and loaded into a Roche GS Flx system using vendor specified chemistries for sequencing. In this study, 210,433 sequence reads, averaging over 13,000 reads per tonsil sample, were generated from 16 tonsil samples from two healthy herds, including the same DNA samples analyzed in the initial study as well as a second set of samples from herd 1 and tonsil brush samples.

3. For the studies in Objective 2, we used 454 Titanium pyrosequencing. All samples were collected with tonsil brushes and DNA extracted. Community template DNAs were amplified with the Broad primers designed for use in the Human Microbiome Project. These primers amplify ~500 bp of sequence covering the V3-V4-V5 region of the 16S rRNA gene. Tonsil brush samples from 43 pigs were analyzed, and ~100,000 sequences generated (~2,400 sequence reads per sample).

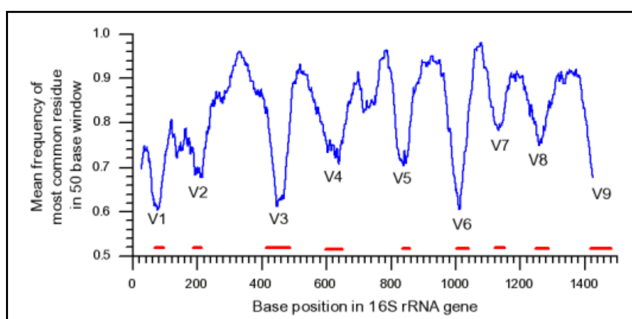


Figure 1. A representation of hypervariable regions within the 16S rRNA gene. The plotted line reflects fluctuations in variability amongst aligned 16S rRNA gene sequences; peaks reflect greater conservation, whilst troughs correspond to the known hypervariable regions V1 to V9 indicated by the red bars. From the bioinformatics Toolkit (www.cardiff.ac.uk/biosi/research/biosoft).

Sequence Analysis Tools

All sequences were processed through the RDP pyrosequencing pipeline [Cole et al, 2005; Cole et al, 2009]. Initial processing included screening and removing short reads (those lacking both primer sequences) and low quality reads (any with errors in the primer sequence). Sequences were sorted based on sequence tags and trimmed of primer and tag sequences. The remaining high quality sequences were taxonomically identified using the Classifier tool at a 60% confidence level. The classifier output was then used for analysis of similarities and difference between herds. For analysis of the data at the genus level, all genera with fewer than 5 representatives were dropped from the analysis.

To identify members of the family *Pasteurellaceae* and genus *Streptococcus* to the lowest possible phylogenetic level, we obtained all the 138 near full-length type sequences from family *Pasteurellaceae* and genus *Streptococcus* from RDP release 10.22 (August 2010). We also added sequence AF486274 ("*Actinobacillus porcitonsillarum*"). These 139 sequences were aligned by the Infernal aligner [Nawroki et al, 2009] trained by RDP [Cole et al, 2009]. The final reference set contained the region corresponding to the 454 FLX amplicon (*E. coli* position 578 to 784) sliced from the alignment. To determine the nearest neighbor, the 454 FLX sequences were aligned by the Infernal aligner and the distance between each FLX sequence and reference sequences was calculated. The reference sequence with the closest distance was reported. In case of tie, all the reference sequences were reported.

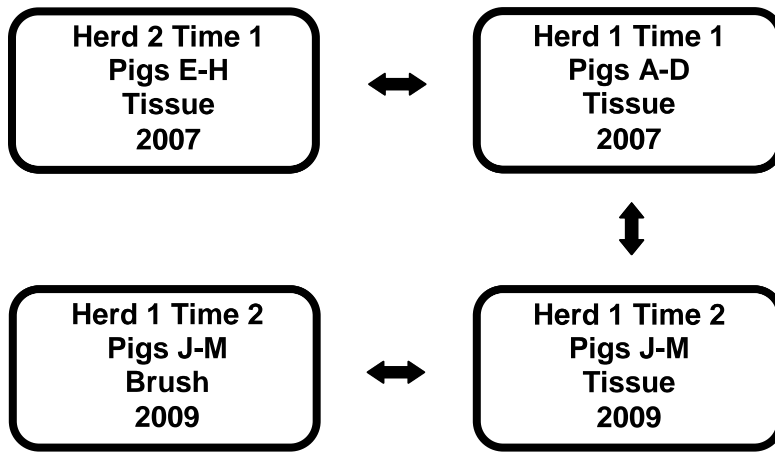
Statistical Analysis

For the statistical analyses of sequences, we used a 0.03% cutoff value for clustering. This is consistent with previous analyses of 454 data [Huse et al, 2010] as well as the historical value frequently used over the past 15 years [Stackebrandt & Goebel, 1994; Rossello-Mora & Amann, 2001]. Similarly we used this cutoff in evaluating members of family *Pasteurellaceae* and genus *Streptococcus*. For comparative statistical analyses, aligned sequences were clustered using the RDP Complete Linkage Clustering Tool and the resulting cluster files were used to calculate Jaccard and Sørensen indices [Cole et al, 2009]. Cluster files were also reformatted with the EstimateS Formatter Tool through the RDP website. Principle component analysis followed by centroid calculations with a 95% confidence limit were performed in R (version 2.10; http://www.r-project.org/) with Vegan package (http://vegan.r-forge.r-project.org) using the EstimateS formatted files. Chao 1 was calculated using the cluster files derived from each sample and from merged samples for herds using the RDP Pyrosequencing Pipeline. Simpson's Diversity index was calculated with MOTHUR [Schloss et al, 2009].

Results:

Objective 1: To determine the composition and structure of the normal microbial community of the tonsils of healthy pigs.

We have completed this objective and the results are in press in the journal BMC Microbiology (preprint attached). In this study, we compared 4 groups of samples: tonsil tissue samples collected in spring 2007 from two different herds, and again in spring 2009 from Herd 1, and tonsil brush samples also collected in spring 2009 from Herd 1, to test the validity of this rapid and painless sampling technique in comparison to euthanasia and removal of tonsil tissue. Sixteen samples (four groups of four samples) were collected and analyzed by 454 Flx pyrosequencing, and comparisons were made between Herd 1 and Herd 2, between Herd 1 Time 1 and Herd 1 Time 2 tissues, and between tissue and brush samples from Herd 1 Time 2 in these results (Fig. 2).



Overall, we found strong similarities between the four groups of samples as well as minor unique differences. We identified a "core microbiome", which we have defined as "found in most animals in all groups", for porcine tonsils that includes eight core genera from six core families (*Pasteurellaceae, Moraxellaceae, Fusobacteriaceae, Veillonellaceae, Peptostrepto-coccaceae,* and *Streptococaceae*) as well as members of the *Enterobacteriaceae*, which varied in genera found from sample to sample, and *Neisseriaceae,* which could not be identified to the genus level (Table 1). *Pasteurellaceae (Actinobacillus, Haemophilus,* and *Pasteurella* species) dominated the tonsillar microbial communities in all pigs examined, comprising on average 60.2% of the total reads, and ranging from 39.2 – 87.0% in individual pigs, which compares well with the previous study using clone libraries [Lowe et al, 2011].

While there were clear and strong similarities between the core microbiomes of all of the groups examined, there were also unique differences in minor genera found or missing from particular groups. For example, reads identified as *Arcanobacterium* were found in all Herd 2 samples, and comprised 0.93% of the reads from that herd, but were not found in any Herd 1 sample. In contrast, reads identified as *Treponema* were found in all but one sample from Herd 1, but not in any sample from Herd 2, and *Chlamydia* were found in Herd 1 tissue samples but not in Herd 2 samples. *Lactobacillus* was abundant in most samples from both Herd 1 time 1 and Herd 2, but was rare in Herd 1 time 2 samples. These results indicate that, despite the small sample number, we can identify differences in the minor genera found in the two different herds.

Figure 3 shows the Jaccard analysis of the clustered sequences from each tonsil community. The samples from Herd 1 and Herd 2 from the same year (Time 1, 2007) are clearly distinguishable. Samples from Herd 1 taken two years later (Time 2, 2009) group with samples taken in time 1 from Herd 1, but are distant from Herd 2. The Jaccard indices of the time 2 sampling from Herd 1 where community samples were derived from both tonsil tissue and brushed tonsils indicate high similarity between these two sampling methods.

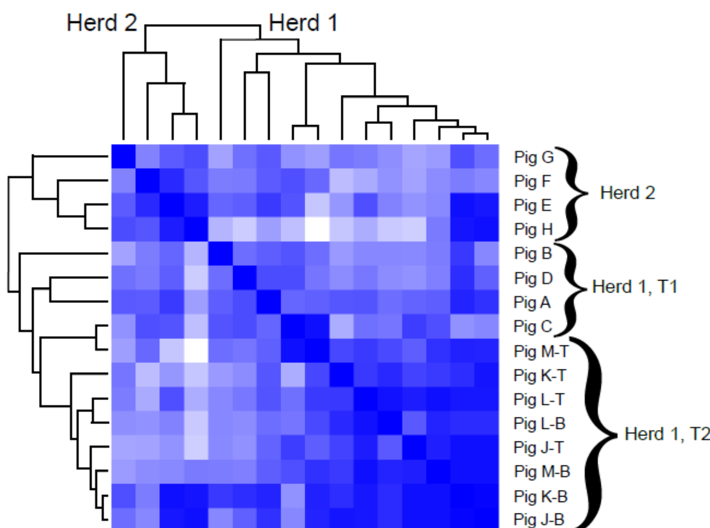


Fig. 3. Jaccard indices of pig tonsil communities. Indices are presented clustered and plotted in heat map format where light to dark indicates increasing similarity.

Principle component analysis (PCA), using the first two factors (PC1 and PC2) was performed using communities from each pig sampled (Figure 4). Each point represents one tonsil community while the colored areas represent the 95% confidence limit of each group. Using the first two components explains 63% of the total variation among the individual samples. This demonstrated that the microbial communities were distinguishable from one another, but relatively close in phylogenetic space as judged by the range of eigenvalues.

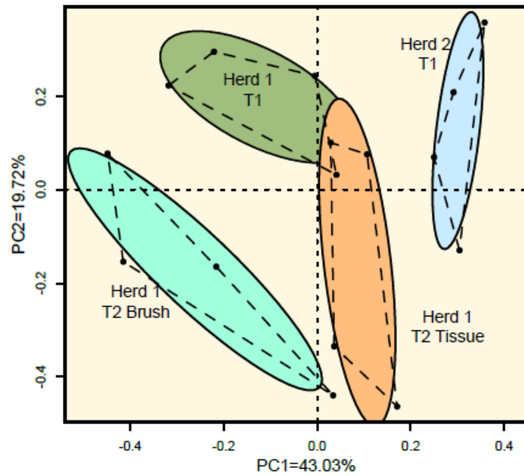


Figure 4: Principle Component Analysis (PCA) results on all individuals sampled. PCA was performed at the level of OTUs, clustering sequences at a 3% difference. The PCA plot of tonsillar communities shows PCA analysis using the first two components, accounting for 62.75% of the sample variation. Each point represents the tonsillar community of one individual pig. Colored circles represent the 95% confidence limit for each group of samples.

A goal of this project was to test tonsil brushes as an alternative, non-invasive method to collect tonsil samples, eliminating the need to euthanize animals to collect tonsil tissue. The Jaccard analysis (Fig. 3) clearly indicated that all samples from the second sampling of Herd 1 were more similar to each other than to samples from Herd 1 and 2. We could detect differences between the brush and tissue extraction procedures as indicated in Figure 4, but the difference was small based on the range of eigenvalues. The detected statistical differences were a consequence of an increase in the percentage of reads identified as *Actinobacillus,* fewer sequences of *Fusobacterium, Veillonella,* and *Peptostreptococcus*), ,and no detectable sequences from the obligate intracellular pathogen *Chlamydia* in the brush specimens. These results indicate that use of tonsil brushes provides a slightly different but sufficiently representative picture of the microbial community that is suitable for within or across herd comparisons when compared to direct extraction of tonsil tissue.

Objective 2: To compare the microbial communities found in healthy pigs to those found in pigs that are carriers of specific porcine pathogens.

The goals of this objective were to utilize the culture-independent techniques optimized in Objective 1 to analyze the microbial communities in the tonsils of herds with disease problems with known bacterial pathogens and to compare the microbiota in tonsils of healthy and carrier/diseased pigs to identify species that differ between these groups. We chose to use tonsil brushes for collection of these samples, since the data generated in Objective 1 indicated that this was a suitable method and this allowed us to collect many more samples than if we had needed to purchase and euthanize pigs for sampling.

Overall, we collected 164 tonsil specimens from 12 herds. In most cases, we collected 5-8 samples from each herd. If possible in the infected herds, we collected samples from both diseased animals and asymptomatic controls. In the case of the herd with *Salmonella*, we collected 50 tonsil brush samples at 10 weeks of age as well as 6 fecal samples at 10, 12, 14, 16, 18, and 20 weeks of age. Once fecal samples had been processed to demonstrate fecal shedding, we chose 6 pigs that shed *Salmonella* in 1-3 fecal specimens and 6 matched pen mates that did not shed *Salmonella* in any of the 6 fecal specimens.

One problem that we found with the 454-FLX pyrosequencing data presented above was that we failed to detect significant numbers of *Bacteroidetes*. In the initial study using clone libraries [Lowe et al 2011], sequences identified as belonging to the order *Bacteroidales* (genera *Bacteroides, Prevotella,* and *Porphyromonas*) comprised the second most dominant group (30% of the sequenced clones) after the *Pasteurellales*, and were found in almost all animals. In contrast, *Bacteroidales* comprised 0.3% of the sequence reads in our 454-FLX pyrosequencing study, including among eight samples that were also used in the clone library study. An unexpectedly low abundance of *Bacteroidetes* has been found in other studies using high-throughput bar-coded pyrosequencing. This discrepancy led us to use a third technique, 454-Titanium pyrosequencing, for analysis of the samples in Objective 2. We first analyzed several of the same

DNA samples used in both prior studies with 454-Titanium primers and pyrosequencing and showed that we could detect *Bacteroidetes* DNA in those samples.

We have completed Titanium sequencing of 41 specimens from 6 herds, as well as controls. These herds included:

IA - a high health status university research herd, located in Iowa
EP - a herd with chronic *Streptococcus suis* disease, Iowa
CK - a herd with chronic carriage and shedding of *Salmonella*, Michigan
- note that this herd is split into *Salmonella* shedders and non-shedders
NC - a herd with an outbreak of *Actinobacillus pleuropneumoniae*, North Carolina
MT - a herd with an outbreak of *Actinobacillus pleuropneumoniae*, Ontario
RM - a herd with an outbreak of *Actinobacillus pleuropneumoniae*, Ontario

A total of 101,031 quality sequencing reads were obtained, averaging ~2,500 reads per sample and ~15,000 reads per herd or group. The number of reads for each individual animal and each group are shown in Table 2 and form the basis of the comparisons of number of OTUs, Chao-I richness, and the Shannon richness and evenness indices. These results indicate that all of the herds except the IA herd had high levels of diversity (Chao-1 ranging from 420 to 924) and richness (Shannon index from 3.0 to 4.27) and were quite uneven (Shannon evenness index of .05 to .69). In contrast, the IA herd showed much lower diversity and higher evenness.

Overall, we found 18 families and 19 genera of bacteria present in all or most pigs in all of the herds (Table 3 and pie charts at end of report). The predominant families included the *Clostridiaceae, Pasteurellaceae, Moraxellaceae, Porphyromonadaceae,* and *Streptococcaceae*. Predominant genera varied between the herds, but the top five included *Clostridium, Actinobacillus, Moraxella, Porphyromonas,* and *Streptococcus.* Distribution of the predominant families and genera for each herd (with the CK herd split into *Salmonella* shedders and non-shedders) are shown in the pie charts at the end of the report.

These results indicate that the composition of the tonsillar bacterial community does indeed vary with the presence of specific pathogens, at least in some cases. For example, in pigs from the EP herd, which had chronic problems with *Streptococcus suis,* the genus *Streptococcus* comprises 20% of the community; in other herds, *Streptococcus* comprises less than 8%. In pigs from the CK herd, which had *Salmonella*, *Enterobacteriaceae* comprise 9% of the tonsillar community in pigs known to shed *Salmonella,* and were not detected in pigs that did not shed *Salmonella*. However, the three herds with *Actinobacillus pleuropneumoniae* problems did not show increased amounts of *Actinobacillus.* We do not yet know whether there was a shift in these pigs from *Actinobacillus* species that are considered to be normal microbiota, such as *A. indolicus, A. minor* and *A, porcitonsillarum*, to *A. pleuropneumoniae*; this will require additional analysis to identify the species present, which can not be done with the programs available in the RDP Pipeline.

We felt it was important to examine not only herd-to-herd variations in the distribution of bacterial OTUs, but also the pig-to-pig variation. Pie charts for all pigs in two herds, the IA herd and the NC herd, illustrating the extremes in pig-to-pig variation that we found, are shown at the end of the report. In the very high health status IA research herd, the communities in the five pigs examined show very little pig-to-pig variation, and are dominated by *Moraxellaceae* (64%, genus *Moraxella*) and *Pasteurellaceae* (28%, 20% genus *Actinobacillus* and 8% unclassified *Pasteurellaceae*), with 5% *Streptococcaceae* (genus *Streptococcus*). In contrast, in the NC herd, an outbreak of *Actinobacillus pleuropneumoniae* began 7-10 days prior to sampling, and all pigs were injected with Excenel (ceftiofur) 1 week prior to sampling. While the overall summary chart looks quite similar to that for all pigs from all herds in this study, there is striking variation in the tonsillar communities in the individual pigs. For example, pigs 1 and 3 contain predominantly *Clostridiaceae* (dark green), which seems to occur in many pigs receiving antibiotics. Pig 6 contains almost entirely *Pasteurellaceae* (red). *Moraxellaceae* (peacock blue) predominates in pig 5, and *Neisseriaceae* (yellow) in pig 2. While this herd shows the most pig-to-pig variation, all of the infected herds showed much more pig-to-pig variation than the high health status herd.

We were surprised to find so many pigs with tonsillar communities dominated by *Clostridiales,* particularly the genus *Clostridium*. The IA herd had ~0.5% *Clostridiaceae*, and the EP herd averaged 8.9% *Clostridiaceae*. In contrast, both the RM herd and the CK *Salmonella* non-shedders had >75% *Clostridiales* overall. Individual pigs in the NC, RM, MT, and CK herds ranged as high as 81.3% *Clostridiaceae*. All four of these herds had received either a β-lactam antibiotic (the NC pigs were injected with Excenel; the MT pigs has penicillin in feed) or chlortetracycline in feed (CK, RM herds).

Discussion:

The research in this study provides the first detailed culture-independent analysis of the tonsillar microbial community in pigs, and indeed a more in depth analysis that has been done in any mammal. As part of this study, we designed a tonsil brush for use as a specimen collection tool and validated its use as a method to collect specimens from live pigs with minimal distress and no harm, which greatly increased the number of herds and pigs that we could sample. We collected specimens from two high health status herds as well as multiple herds with defined disease caused by bacteria that have the tonsils as a frequent reservoir site. We used sequencing of the conserved bacterial gene encoding 16S ribosomal RNA to characterize which bacteria were present in tonsils, and identified a "core microbiome" found in healthy pigs. We analyzed both herd-to-herd and pig-to-pig variation in these pigs, and demonstrated that the bacterial communities in the healthy herds examined showed strong similarities in the major components as well as distinct differences in the minor components. This work has been published in two manuscripts.

We hypothesized that the microbial communities in pig tonsils would vary in pigs from herds with disease caused by pathogens that frequently reside in the tonsils. Our results demonstrate that this is true, at least in some herds. The data on other herds was confounded by the use of antibiotics. In some herds, it is difficult to separate the effect of antibiotics from the effect of a pathogen. It is very interesting to see that use of antibiotics, either β-lactams or chlortetracycline, seems to lead to an increase in the clostridia present in tonsils. This parallels what is known about effects of antibiotics on the microbiota of the gastrointestinal tract, where antibiotics often lead to both disruption of the normal microbiota and an increase in clostridia, such as *Clostridium difficile*, the cause of antibiotic-associated pseudomembranous colitis in humans and some animals. In humans, long term use of low levels of antibiotics can lead to increased susceptibility to gastrointestinal pathogens such as *Salmonella*. It would be very interesting to pursue how antibiotics affect the normal tonsillar microbiota in a healthy herd, and whether this leads to an increased susceptibility to colonization by pathogens and subsequent disease.

In this study, the methods used to sequence the amplified community 16S rRNA genes evolved. In our initial study, we used amplified community DNA and cloned the amplicons into plasmid vectors to construct clone libraries, and then sequenced ~ 500 bp from each clone. This expensive and labor-intensive process provided at best 96-192 clones per sample, enough to identify only the most common organisms in the sample. In addition, we found that the universal primers used for PCR amplification and sequencing were biased against Gram-positive organisms, leading to under-identification of clostridia, streptococci, and staphylococci. As technology improved, we switched to 454-FLX pyrosequencing. This method allowed us to sequence thousands of 16S amplicons per sample, giving great depth to the view of the microbial communities. However, the short length of sequence generated, roughly 150 bp, limited the ability to identify sequences to the genus level. Further, we found that the primer set routinely used for this method failed to amplify *Bacteroidetes* (*Bacteroides, Porphyromonas,* and *Prevotella*), thus almost eliminating detection a group that we had found to comprise 30% of the 16S rRNA gene amplicons in the clone library. This result led us to switch to a third sequencing technique, Titanium pyrosequencing, which generates 400-500 bp sequences that at this point do not seem to be biased for or against any particular bacterial group. With this technique, we were able to generate ~2500 sequences per sample, which provides sufficient depth to detect bacteria that comprise 0.1% of the community. All of the results in Objective 2 were generated with Titanium sequencing.

We plan additional analysis of the data to attempt to identify bacterial present in the samples to the species level. We were able to do this with a subset of the data in the Lowe et al, 2012 manuscript, using a computer program written specifically for that study. We expect to be able to do the same with the data from the Titanium sequencing, or at least some of it, once we can generate the required program. This can not be done using the standard sequence analysis tools available through the RDP Pipeline. That data may reveal species present only when a specific pathogen is present, or species whose presence seems to preclude the pathogen, which might be developed into a probiotic.

As we originally proposed, this research has produced the first in depth **culture-independent** characterization of the microbial community in the tonsils of pathogen-free pigs and pathogen carriers and of the level of community heterogeneity from pig-to-pig and herd-to-herd. It has provided a first glimpse at differences between microbial communities found in healthy pigs and pathogen carriers. It has not as yet identified organisms with the potential for development as probiotics to inhibit colonization with specific pathogens and reduce disease incidence without the use of antibiotics, or sentinel organisms whose presence is a cue that a herd is susceptible to a particular pathogen, but has laid the groundwork for subsequent studies

that may identify such organisms. This research has made a solid start towards a better understanding of the interactions between normal microbiota and pathogens within the porcine upper respiratory tract that allow establishment and maintenance of carriage of pathogens and development of systemic disease.

Table 1: The core microbiome of porcine tonsils

Phylum	% of total	Class	% of total	Order	% of total	Family	% of total	Genus	% of total
Proteobacteria	73.4	*Gammaproteobacteria*	69.8	*Pasteurellales*	56.0	*Pasteurellaceae*	60.2	*Actinobacillus*	37.0
								Haemophilus	6.6
								Pasteurella	16.1
				Pseudomonadales	11.8	*Moraxellaceae*	12.3	*Alkanindiges*	12.0
				Enterobacteriales	2.0	*Enterobacteriaceae*	2.2		
		Betaproteobacteria	3.2	*Burkholderiales*	0.3				
				Neisseriales	2.8	*Neisseriaceae*	3.0		
		Alphaproteobacteria	0.3						
Firmicutes	17.8	*Clostridia*	14.3	*Clostridiales*	14.3	*Peptostreptococcaceae*	2.2	*Peptostreptococcus*	2.6
						Veillonellaceae	4.4	*Veillonella*	3.2
		Bacilli	3.5	*Lactobacillales*	3.4	*Streptococcaceae*	0.5	*Streptococcus*	0.6
Fusobacteria	5.6	*Fusobacteria*	5.6	*Fusobacteriales*	5.6	*Fusobacteriaceae*	5.6	*Fusobacterium*	7.0
Actinobacteria	1.2	*Actinobacteria*	1.2	*Actinomycetales*	0.9				
Bacteroidetes	0.8	*Bacteroidia*	0.3	*Bacteroidales*	0.3				
5/17 phyla identified	98.8	8/27 classes identified	98.2	10/34 orders identified	97.4	8/61 families identified	90.4	8/101 genera identified	85.1

NOTE: Almost half of the *Clostridiales* could not be assigned at the family level, and >92% of the *Neisseriaceae* could not be assigned to a genus.

Table 2: Diversity and richness of the tonsillar microbial communities

Sample Name	Description	Number of Sequences	Number of Clusters	Chao 1 Estimate	Shannon diversity	Shannon evenness
ISU7	High health status	2592	49	68	0.92	0.24
ISU8	High health status	1739	29	36	1.15	0.34
ISU9	High health status	2687	59	87	1.67	0.41
ISU10	High health status	2308	44	77	0.98	0.26
ISU11	High health status	3672	75	101	1.59	0.37
ISU Herd	High health status	12998	159	214	1.76	0.35
EP1	*Streptococcus suis*	2698	119	158	2.82	0.59
EP3	*Streptococcus suis*	8811	360	460	3.72	0.63
EP4	*Streptococcus suis*	1990	70	93	2.34	0.55
EP5	*Streptococcus suis*	3169	97	140	2.46	0.54
EP6	*Streptococcus suis*	2108	94	116	1.93	0.42
EP7	*Streptococcus suis*	7516	516	671	4.50	0.72
EP Herd carriers	*Streptococcus suis*	26292	578	737	3.89	0.63
CK703	*Salmonella*	2150	120	203	2.46	0.51
CK720	*Salmonella*	1469	120	201	2.94	0.61
CK725	*Salmonella*	1511	184	266	3.50	0.67
CK726	*Salmonella*	2546	259	338	4.24	0.76
CK728	*Salmonella*	1326	86	113	2.44	0.55
CK750	*Salmonella*	1871	204	294	3.91	0.73
CK Herd shedders	*Salmonella*	10873	585	762	4.40	0.69
CK704	*Salmonella*	3839	112	139	3.05	0.65
CK719	*Salmonella*	2124	87	120	1.94	0.43
CK724	*Salmonella*	1733	76	105	1.81	0.42
CK727	*Salmonella*	2886	89	128	1.55	0.34
CK730	*Salmonella*	1862	213	296	3.33	0.62
CK749	*Salmonella*	1577	227	356	4.35	0.80
CK Herd: non-shedders	*Salmonella*	14021	537	733	3.48	0.55
NC1	*A. pleuropneumoniae*	2381	102	135	2.54	0.55
NC2	*A. pleuropneumoniae*	2299	71	94	2.63	0.62
NC3	*A. pleuropneumoniae*	1501	63	84	2.47	0.60
NC4	*A. pleuropneumoniae*	2137	126	188	3.53	0.73
NC5	*A. pleuropneumoniae*	1960	88	115	2.84	0.64
NC6	*A. pleuropneumoniae*	2221	24	39	0.48	0.15
NC Herd	*A. pleuropneumoniae*	10278	286	420	3.57	0.63
MT1	*A. pleuropneumoniae*	2045	276	453	4.68	0.83
MT2	*A. pleuropneumoniae*	1864	112	143	2.72	0.58
MT3	*A. pleuropneumoniae*	2279	59	91	2.08	0.51
MT5	*A. pleuropneumoniae*	1528	144	231	3.22	0.65

MT6	*A. pleuropneumoniae*	1955	311	502	4.48	0.78
MT7	*A. pleuropneumoniae*	2264	160	256	2.99	0.59
MT Herd	*A. pleuropneumoniae*	11935	665	924	4.27	0.66
RM1	*A. pleuropneumoniae*	4256	67	77	1.53	0.36
RM2	*A. pleuropneumoniae*	1580	55	80	1.93	0.48
RM3	*A. pleuropneumoniae*	3112	73	113	1.94	0.45
RM4	*A. pleuropneumoniae*	1905	105	160	2.92	0.63
RM5	*A. pleuropneumoniae*	1825	185	251	4.13	0.79
RM6	*A. pleuropneumoniae*	1956	42	54	1.52	0.41
RM Herd	*A. pleuropneumoniae*	14634	372	481	3.00	0.51

Number of clusters = the actual number of .(operational taxonomic units (OTUs) in each sample and herd, based on an 0.03% cutoff = detected richness.

Chao-1 estimates the richness of an environment, i.e., the total number of operational taxonomic units present = estimated richness.

Shannon richness, computed using the RDP Pyrosequencing Pipeline; the higher the number, the greater the diversity of OTUs in the sample.

Shannon evenness: the lower the number, the more even the communities.

Table 3: Most common families and genera found in porcine tonsils

Order	Family	% of total	Genus	% of total
Bacteroidales	Bacteroidaceae	3.10%	Bacteroides	3.4
	Porphyromonadaceae	7.50%	Porphyromonas	7.9
	Prevotellaceae	2.38%	Prevotella	2.2
Clostridiales	Clostridiaceae	23.15%	Clostridium	25.9%
			Sporacetigenium	1.2
	Lachnospiraceae	1.30%	Roseburia	0.4
	Peptostreptococcaceae	4.78%	Peptostreptococcus	0.1
	Ruminococcaceae	1.60%	Faecalibacterium	0.5
	Veillonellaceae	1.73%	Veillonella	1.3
Enterobacteriales	Enterobacteriaceae	1.06%		
Erysipelotrichales	Erysipelotrichaceae	0.83%	Turicibacter	1.0
Flavobacteriales	Flavobacteriaceae	1.28%		
Fusobacteriales	Fusobacteriaceae	1.64%	Fusobacterium	2.1
Lactobacillales	Lactobacillaceae	0.36%	Lactobacillus	1.4
	Streptococcaceae	6.94%	Streptococcus	7.9
Pseudomonadales	Moraxellaceae	11.93%	Moraxella	11.3
			Acinetobacter	0.7
Mycoplasmatales	Mycoplasmataceae	0.75%	Mycoplasma	0.9
Neisseriales	Neisseriaceae	3.21%		
Pasteurellalea	Pasteurellaceae	20.53%	Actinobacillus	15.6
			Haemophilus	0.2
			Pasteurella	1.2
	Other	3.96		

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Lowe, B. A., T. L. Marsh, N. Isaacs-Cosgrove, R. N. Kirkwood, M. Kiupel, and M. H. Mulks. 2012. Defining the "core microbiome" of the microbial communities in the tonsils of healthy pigs. BMC Microbiology, in press.

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X. Publications generated from this research to date:

Lowe, B. A., T. L. Marsh, N. Isaacs-Cosgrove, R. N. Kirkwood, M. Kiupel, and M. H. Mulks. 2011. Microbial communities in the tonsils of healthy pigs. Veter. Microbiol.147:346-57.

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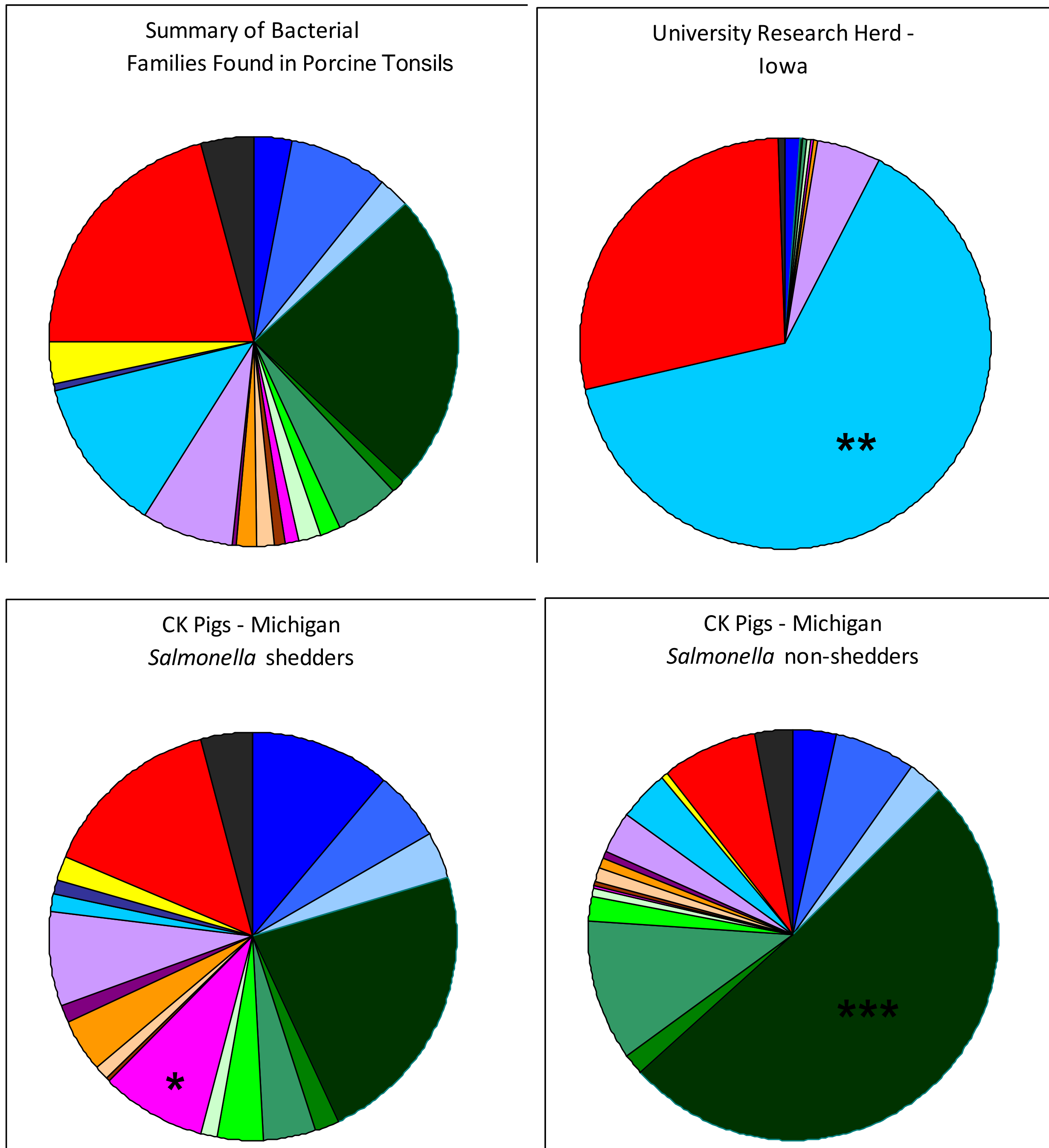
Presentations at conferences:

Mulks, M. H., T. L. Marsh, B. Lowe, N. Isaacs-Cosgrove, and R. Kirkwood. 2009. Microbial ecology of the porcine tonsils. 90th Conf. Res. Workers Anim. Dis., Chicago, IL, Dec. 2009.

Mulks, M. H., B. A. Lowe, T. L. Marsh, N. Isaacs-Cosgrove, R. M. LeVeque, and R. N. Kirkwood. 2011. *Pasteurellaceae* predominate in porcine tonsils. International Pasteurellaceae Society Meeting, Helsingor, Denmark, August 2011.

Mulks, M. H., B. A. Lowe, T. L. Marsh, N. Isaacs-Cosgrove, R. M. LeVeque, and R. N. Kirkwood. 2011. *Pasteurellaceae* predominate in porcine tonsils. Midwest Microbial Pathogenesis Conference, Ann Arbor, MI, October 2011.

Families of Bacteria Found in Porcine Tonsils



- Bacteroidaceae
- Porphyromonadaceae
- Prevotellaceae
- Clostridiaceae
- Lachnospiraceae
- Peptostreptococcaceae
- Ruminococcaceae
- Veillonellaceae
- Enterobacteriaceae
- Erysipelotrichaceae
- Flavobacteriaceae
- Fusobacteriaceae
- Lactobacillaceae
- Streptococcaceae
- Moraxellaceae
- Mycoplasmataceae
- Neisseriaceae
- Pasteurellaceae
- Other

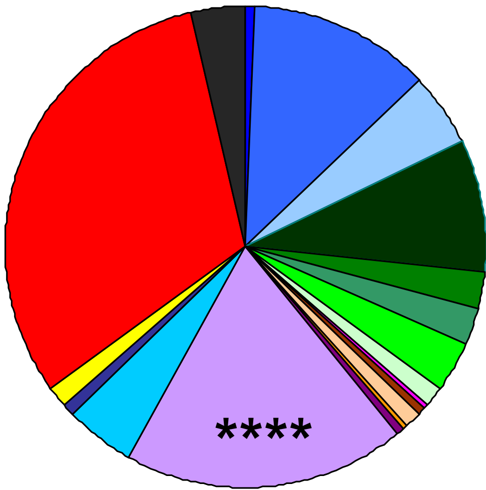
NOTE: Bacillales are in shades of blue; Clostridiales in shades of green; and Lactobacillales in shades of purple

* indicates increased Enterobacteriaceae in *Salmonella* shedders in this herd

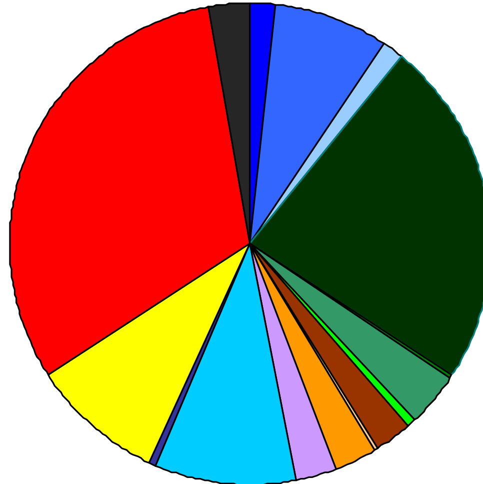
** indicates high level of Moraxellaceae in this very clean research herd

Families of Bacteria Found in Porcine Tonsils

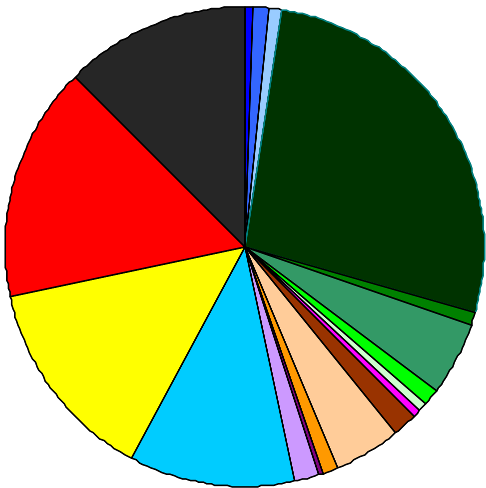
EP Pigs - Iowa
Chronic *Streptococcus suis*



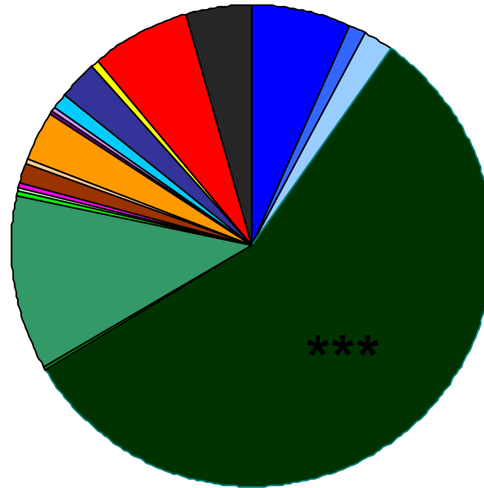
NC Pigs - North Carolina
A. pleuropneumoniae outbreak



MT Pigs - Ontario
A. pleuropneumoniae outbreak



RM Pigs - Ontario
A. pleuropneumoniae outbreak

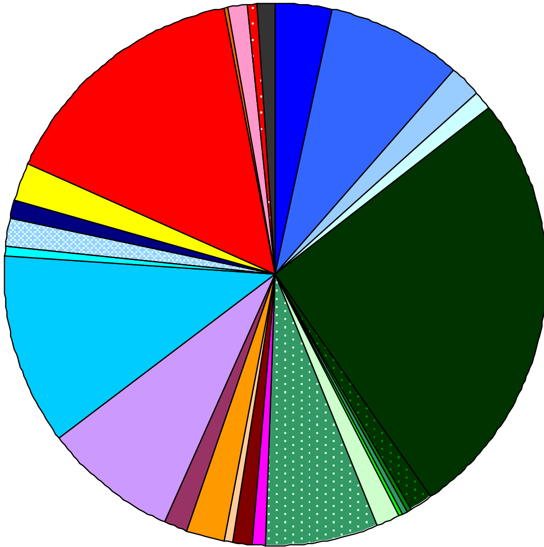


Bacteroidaceae	Porphyromonadaceae	Prevotellaceae
Clostridiaceae	Lachnospiraceae	Peptostreptococcaceae
Ruminococcaceae	Veillonellaceae	Enterobacteriaceae
Erysipelotrichaceae	Flavobacteriaceae	Fusobacteriaceae
Lactobacillaceae	Streptococcaceae	Moraxellaceae
Mycoplasmataceae	Neisseriaceae	Pasteurellaceae
Other		

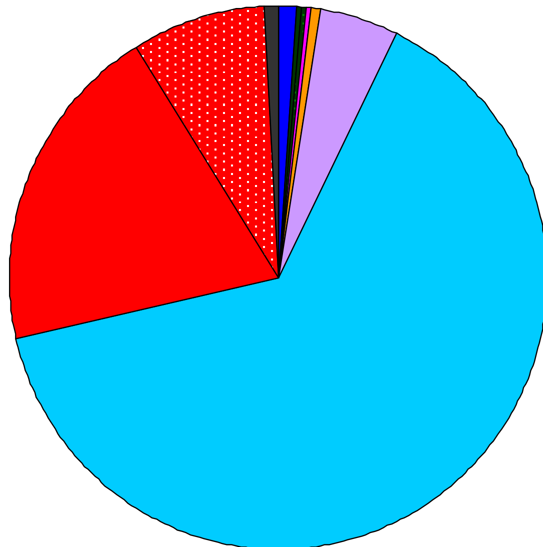
*** indicates very high levels of Clostridiaceae in these two herds
**** indicates increased Streptococcaceae in this herd with chronic *S. suis* disease

Genera of Bacteria Found in Porcine Tonsils

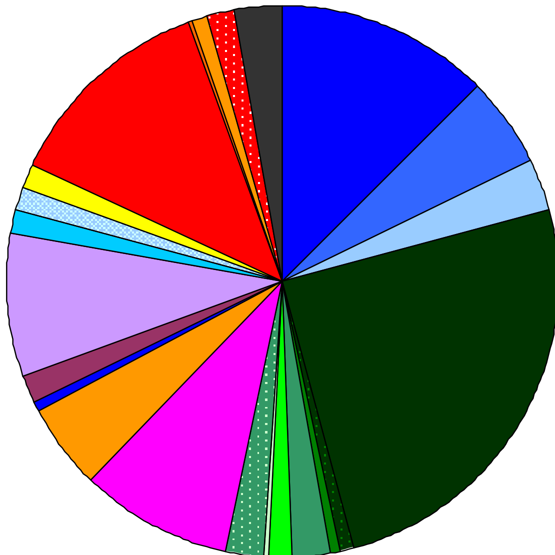
Summary of Bacterial Genera Found in Porcine Tonsils



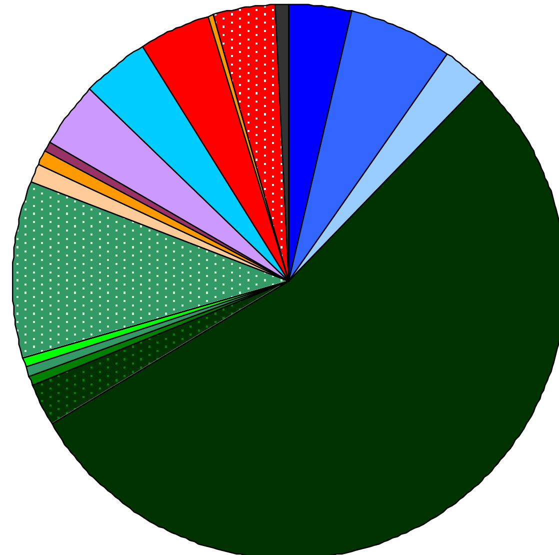
University Research Herd Iowa



CK Pigs - Michigan *Salmonella* shedders



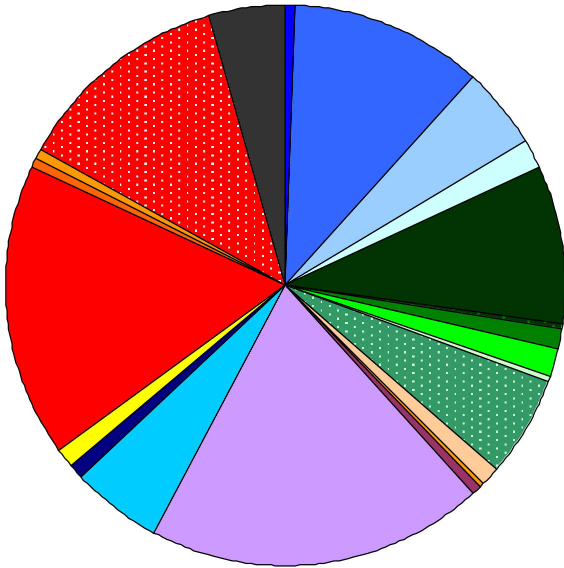
CK Pigs - Michigan *Salmonella* non-shedders



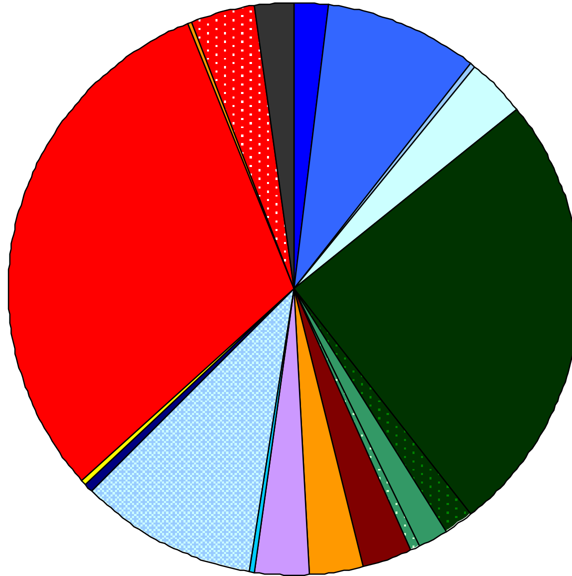
Bacteroides	Porphyromonas	Prevotella
Unclasc. Bacteroidales	Clostridium	Sporacetigenium
Roseburia	Peptostreptococcus	Faecalibacterium
Veillonella	Unclass. Clostridiales	Unclass. Enterobacteriaceae
Turicibacter	Unclass. Flavobacteriaceae	Fusobacterium
Leptotrichia	Lactobacillus	Streptococcus
Moraxella	Acinetobacter	Unclass. Moraxellaceae
Mycoplasma	Unclass. Neisseriaceae	Actinobacillus
Haemophilus	Pasteurella	Unclass. Pasteurellaceae
Other		

Genera of Bacteria Found in Porcine Tonsils

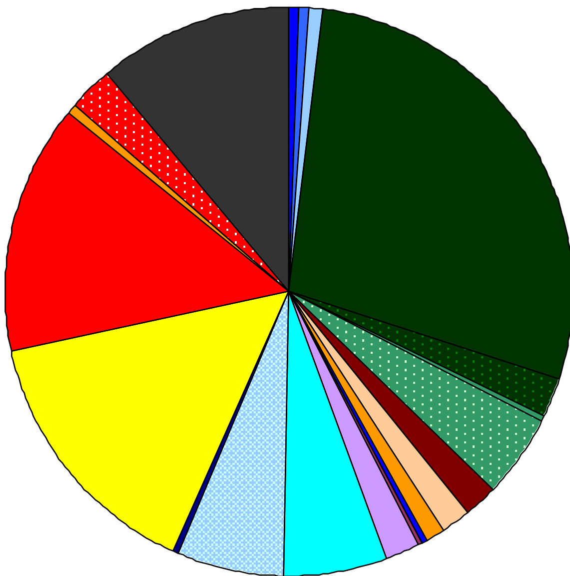
EP Pigs - Iowa
Chronic *Streptococcus suis*



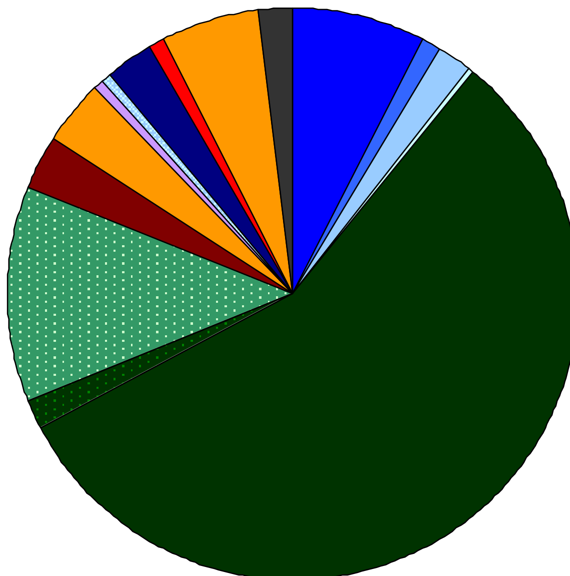
NC Herd - North Carolina
***A. pleuropneumoniae* Outbreak**

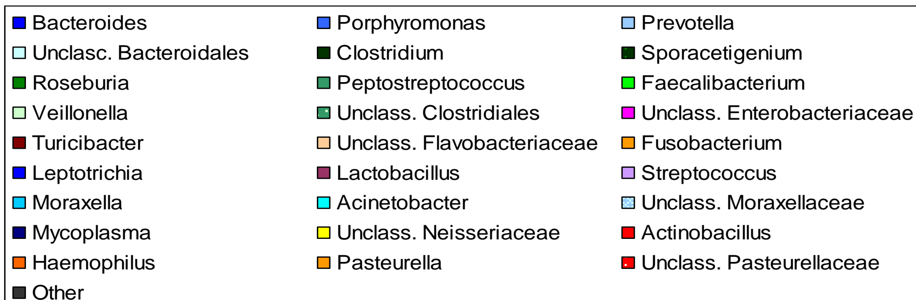


MT Herd - Ontario
***A. pleuropneumoniae* Outbreak**

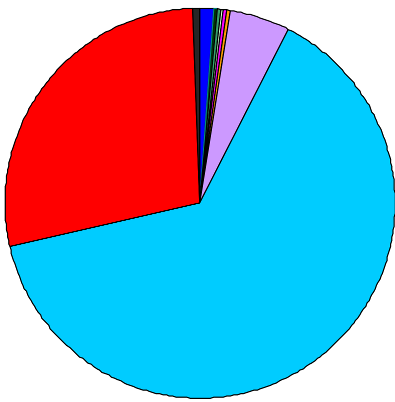


RM Herd - Ontario
***A. pleuropneumoniae* Outbreak**

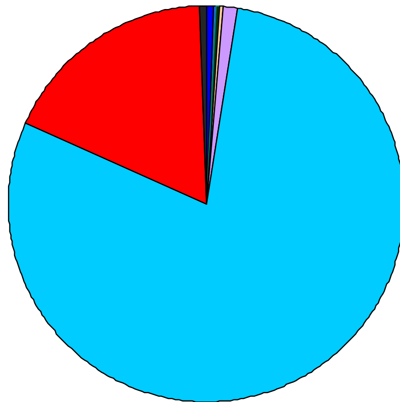




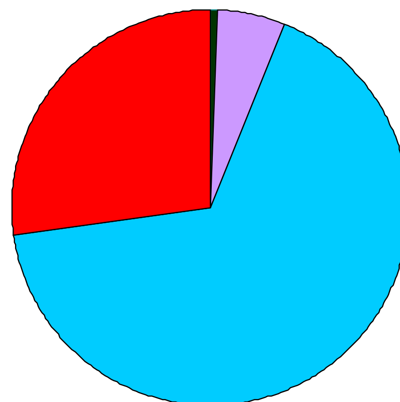
Pig-to-pig Variation in Families of Bacteria Found in Porcine Tonsils



Summary of all 6 pigs



Pig 7



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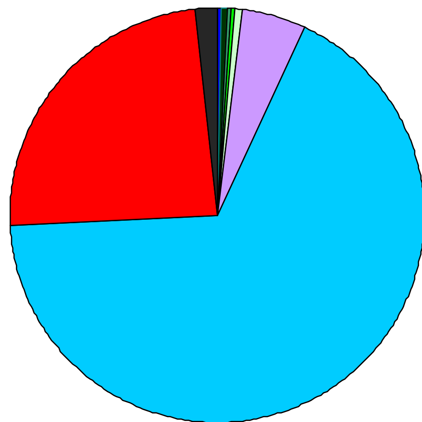

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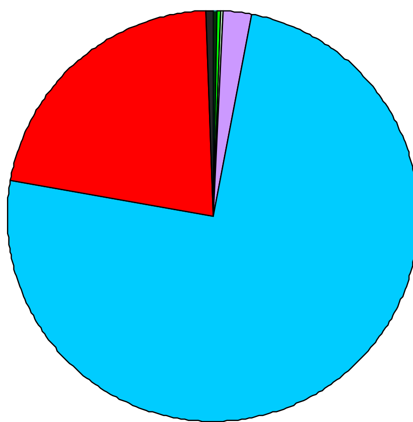

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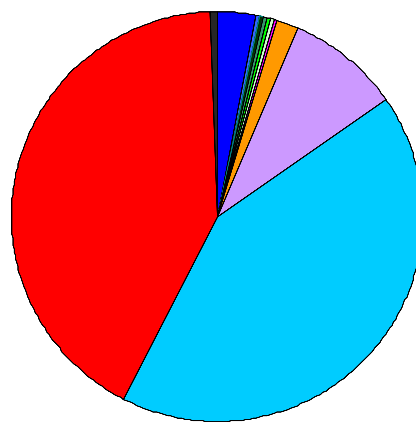

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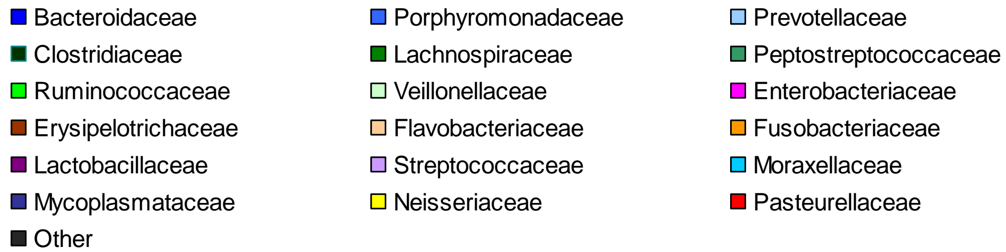
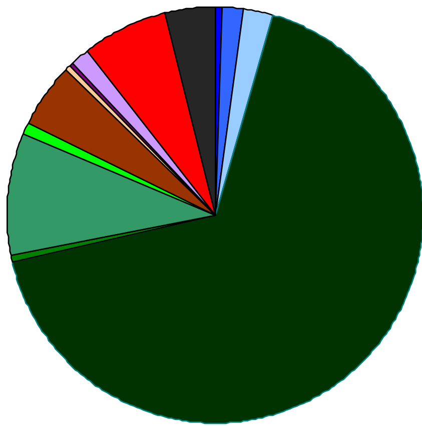
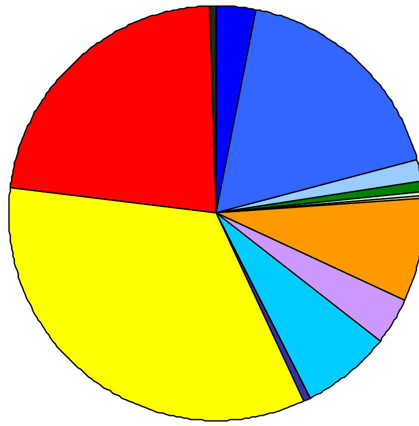


In this very high health status ISU research herd, the communities in the five pigs examined show very little pig-to-pig variation, and are dominated by *Moraxellaceae* (64%, genus *Moraxella*) and *Pasteurellaceae* (28%, 20% genus *Actinobacillus* and 8% unclassified *Pasteurellaceae*), with 5% *Streptococcaceae* (genus *Streptococcus*).

Pig-to-pig Variation in Families of Bacteria Found in Porcine Tonsils



Pig 1



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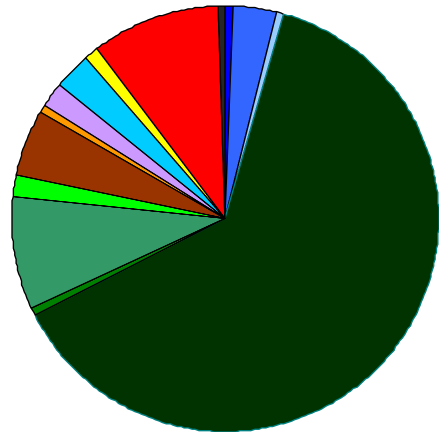
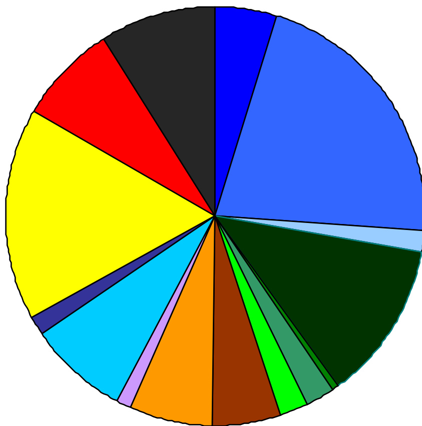


Pig 3



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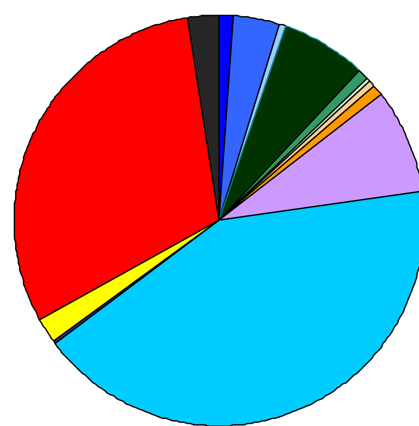
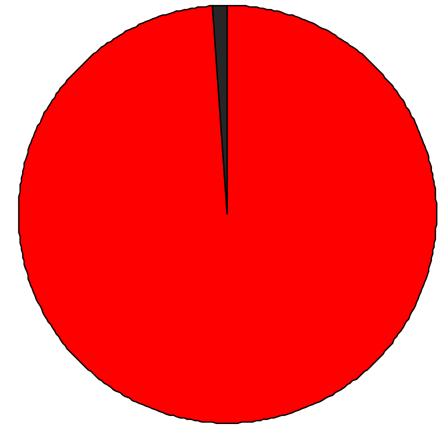
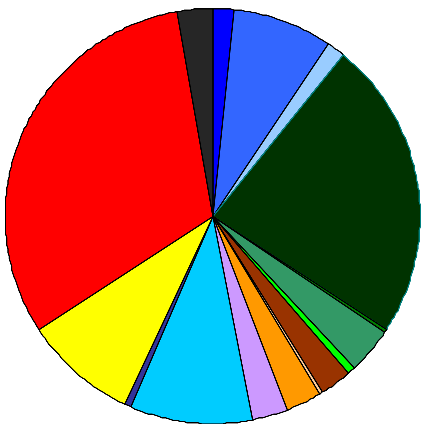


Pig 5



Pig 6



Summary of all 6 pigs

In this North Carolina (NC) herd, an outbreak of *Actinobacillus pleuropneumoniae* began 7-10 days prior to sampling, and all pigs were injected with Excenel (ceftiofur) 1 week prior to sampling. While the overall summary chart looks quite similar to that for all pigs from all herds in this study, there is striking variation in the tonsillar communities in the individual pigs. For example, pigs 1 and 3 contain predominantly *Clostridiaceae* (dark green), which seems to occur in many pigs receiving antibiotics. In contrast, pig 6 contains almost entirely *Pasteurellaceae* (red). *Moraxellaceae* (peacock blue) predominates in pig 5, and *Neisseriaceae* (yellow) in pig 2.