

SWINE HEALTH

Title: Development of a surveillance system to monitor the genetic variability and molecular epidemiology of swine bacterial pathogens – NPB #06-030

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II. Industry Summary:

The **objective** of this project was to develop a surveillance system to monitor the genetic variability and molecular epidemiology of swine bacterial pathogens. The **specific aims** of this projects were: 1) to develop and validate genotyping techniques for *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, and *Actinobacillus suis*; 2) to create a genomic fingerprint database, and 3) to develop an online identification, reporting, and sharing system to facilitate the use of information stored in the genomic fingerprint database by swine veterinarians and other laboratories. The surveillance system created for swine bacterial pathogens is an important tool that veterinarians and producers can use directly for disease control programs. Bacterial genotyping is now offered as a service to field veterinarians at the University of Minnesota Veterinary Diagnostic Laboratory. Genotyping information can be used in several ways: 1) to identify prevalent strains causing disease in specific herds, 2) to select strains to be included in autogenous (or universal) vaccines, 3) to identify new virulent strains introduced into the herd, and 4) to track potential sources of these virulent strains. Field veterinarians can now use the online database to obtain additional information beyond the genotype level regarding specific strains. For example, the database will include the isolation date, the age of the affected pig, the tissue from which it was isolated and the serotype of that particular strain. Each bacterial strain and herd of origin was assigned a unique code for confidentiality purposes. In sum, this database is an important resource for sorting through the epidemiology of swine bacterial pathogens which can be applied directly for disease control and eradication.

The genomic database for swine bacterial pathogens is available for consultation at: <http://molecularbacteriology.com/database.aspx> and is maintained by Dr. Simone oliveira (oliv0107@umn.edu).

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III. Scientific Abstract:

The **objective** of this project was to standardize genotyping techniques for the major bacterial pathogens affecting the swine industry. Additionally, we created a surveillance system and a genomic database that contains the genotype, date of isolation, geographical location, age of affected animals, tissue, lesions associated with isolation, (sero)type, and antibiotic resistance profile. This project was extended until 2008 and expanded to include additional bacterial isolates. **Methods:** Genomic fingerprints were obtained for the following pathogens using the repetitive-element-based PCR (Rep-PCR): *S. suis* (n=140), *A. suis* (n=104), *A. pleuropneumoniae* (n=34). Each bacterial population structure and diversity was evaluated using computer-based analysis. Dendrograms identifying groups of related strains were constructed for each pathogen and the diversity of each species was calculated using the Simpson's index of diversity. **Results:** Primers targeting different repetitive elements were used, including BoxA, Enterobacterial Repetitive Intergenic Consensus (ERIC), and Repetitive element Consensus (REP). Best results were obtained using ERIC and BoxA primers. Results using the REP primers were inconsistent and this primer was not used in the analysis. Better reproducibility was obtained using the highest annealing temperature tested (50°C) for both primers, and further analysis was performed using this temperature. Diversity and discriminatory power were defined using reference strains and field isolates for each of the pathogens tested. The diversity index for *A. pleuropneumoniae* reference strains was D=0.96 for the ERIC-PCR, D=0.71 for the Box-PCR, and D=0.96 for the combined dendrogram based on both genotyping methods. For *Actinobacillus suis*, the diversity index was D=0.64 for ERIC-PCR, D=1.00 for the BOX-PCR, and D=1.00 for the combined dendrogram. For *Streptococcus suis*, the ERIC-PCR diversity index was D=0.93, the Box-PCR index was D=0.92, and the combined dendrogram had a diversity index of D=0.97. These experiments demonstrated the need to standardize and validate each of these techniques for each of the organisms tested. Following this analysis, further testing of *A. pleuropneumoniae* field isolates was performed by ERIC-PCR, whereas BOX and ERIC-PCR were used to genotype *A. suis* and *S. suis* isolates. Serotyping was also used to define *S. suis* strains groups, as the dendrogram containing ERIC-PCR, BOX-PCR, and serotyping increased the diversity index to D=0.98. Dendrograms containing the most relevant pathotypes were constructed and are available for consultation at the site www.molecularbacteriology.com.

IV. Introduction:

Molecular epidemiology can be defined as the use of molecular-based tools in epidemiological studies. Gene sequencing has been largely used as the gold standard to study strain variation for many viruses, such as PRRSV¹ and SIV², for example. Viruses usually have very small genomes, which makes it easier to select a specific gene to be used as a marker for epidemiological studies. Bacterial genomes are much larger and more complex than viral genomes. The genome size can vary from one to six million base pairs, which is 66 to 400 times the size of PRRSV genome, for example. Sequencing a single gene (or a fragment of a gene) from a bacterial genome is unlikely to represent the variability present in the whole genome. That is why methods that can characterize large portions of the genome are preferable to study the molecular epidemiology of bacterial pathogens.

There are several methods that can be used to obtain what is called a “fingerprint” of the bacterial genome.³ These methods vary in differentiation power and, more importantly, they vary in the workload involved to obtain results. Pulsed Field Gel Electrophoresis (PFGE), for example, has been used as the gold standard for bacterial genotyping since it was first described in the early 80’s.⁴ However, this technique is too expensive and timing-consuming for routine use. A PCR-based method called Rep-PCR, which allows typing of bacterial organisms in a single PCR reaction, was developed in the early 90’s. Primers used in the Rep-PCR reaction are complementary to repetitive sequences in the bacterial DNA, which were found to be highly conserved among different bacterial species.⁵ Since its initial development, the repetitive element-based PCR has been widely used to genotype human and animal bacterial pathogens.

Currently, there are no surveillance systems available to monitor diseases caused by swine bacterial pathogens in the US. Genotyping has been validated for *Haemophilus parasuis*,⁶ but not for other respiratory microorganisms such as *Streptococcus suis*, *Actinobacillus suis*, or *Actinobacillus pleuropneumoniae*. These agents are frequently involved in clinical disease episodes with high mortality in naïve herds, and genotyping would be an excellent tool to study the epidemiology of infection by these organisms. As demonstrated for *H. parasuis*^{6,7} genotyping data can be used to define prevalent strains causing mortality, to select strains to be included in autogenous vaccines, to identify new strains introduced into the herd, and to track potential sources of virulent strains. This information can be used to improve control and biosecurity protocols, and this concept can be applied to other bacterial pathogens.

References

1. Dee SA, Torremorell M, Rossow K, Mahlum C, Otake S, Faaberg K. (2001) Identification of genetically diverse sequences (ORF 5) of porcine reproductive and respiratory syndrome virus in a swine herd. *Can J Vet Res.* 65(4):254-60.
2. Choi YK, Goyal SM, Joo HS. (2002) Prevalence of swine influenza virus subtypes on swine farms in the United States. *Arch Virol.* 147(6):1209-20.
3. Versalovic J, Lupski JR. (2002) Molecular detection and genotyping of pathogens: more accurate and rapid answers. *Trends Microbiol.* 10 (10 Suppl):S15-21.
4. Olson MV. (1989) Separation of large DNA molecules by pulsed-field gel electrophoresis. A review of the basic phenomenology. *J Chromatogr.* 470(2):377-83.
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6. Oliveira S, Blackall P, Pijoan C. (2003) Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. *Am J Vet Res.* 64(4):435-42.
7. Oliveira S, Pijoan C. (2004) *Haemophilus parasuis*: new trends on diagnosis, epidemiology and control. *Vet Microbiol.* 99(1):1-12.

V. Objectives

To develop a surveillance system to monitor the genetic variability and molecular epidemiology of swine bacterial pathogens by the following steps:

1. Develop and validate genotyping techniques for *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, and *Actinobacillus suis*.
- 2.
3. Create a genomic fingerprint database for *S. suis*, *A. pleuropneumoniae*, *A. suis*, and *H. parasuis*.
- 4.
5. Develop an online identification, reporting, and sharing system to facilitate the use of information stored in the genomic fingerprint database by swine veterinarians and laboratories.

VI. Materials & Methods:

Experimental design: Genotyping of *Haemophilus parasuis* has been previously validated by the principal investigator.¹ Reference strains and field isolates for *S. suis*, *A. pleuropneumoniae*, and *A. suis* were used to develop and validate the genotyping technique for these organisms. Different primers and PCR conditions were evaluated to optimize the discriminatory power of the technique for each bacterial species. Genetic variability analysis was performed using the BioNumerics V.5.1 software. Relevant clusters of strains were defined based on the calculation of point-bisectional correlation (statistical function of the software).³ As part of the surveillance program, each strain was assigned a unique identification code. A genomic fingerprint database was created for each bacterial species. The genomic fingerprint database is available online for consultation by field veterinarians and other laboratories (www.molecularbacteriology.com). Bacterial genotyping, analysis of genetic variability, and construction of dendrograms is now offered as a service to field veterinarians.

Bacterial strains: Reference and field strains for *A. pleuropneumoniae*, *S. suis*, and *A. suis* were obtained from the American Type Culture Collection (<http://www.atcc.org/>) and from the MVDL. These strains were selected from different geographical regions and unrelated herds.

Rep-PCR: Rep-PCR refers to repetitive element based-PCR. There are several repetitive sequences that can be used for bacterial genotyping. REP (Repetitive Extragenic Palindrome or REP-PCR), ERIC (Enterobacterial Repetitive Intergenic Consensus or ERIC-PCR) and Box-A element (described for *Streptococcus pneumoniae* or Box-PCR) are the most used for bacterial genotyping. A modification of the protocol described for *H. parasuis* genotyping by Oliveira et al (2003) was used.¹ In order to define the best set of primers and PCR conditions that generated the maximum diversity index, the following primers and annealing temperatures were tested for each bacterial species:

Organisms	Primers*	Identification	Sequence	Annealing**
<i>S. suis</i>	BOX A1R	Fw/ Rv	5'-CTACggCAAggCgACgCTgACg-3'	40-50 °C
	ERIC 1R	Forward	5'-ATgTAAgCTCCTggggATTCAC-3'	40-50 °C
<i>A. pleuropneumoniae</i>	ERIC 2	Reverse	5'-AAgTAAgTgACTggggTgAgCg-3'	-
<i>A. suis</i>	REP 1R	Forward	5'-IIIIcGlCgICATClggC-3'	40-50 °C
	REP 1R	Reverse	5'-ICgICTTATClggCCTAC-3'	-

*Reference for primer sequences: J.L.W. RADEMAKER¹ and F.J. DE BRUIJN^{1,2,3}. ¹MSU-DOE Plant Research Laboratory, ²Department of Microbiology, ³NSF Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824, USA

** Gradual increases of 2°C in the annealing temperature within the interval from 40 to 50°C will be tested, totaling 8 different annealing temperatures per bacterial species.

Criteria for selection of final PCR conditions: Selection of ideal PCR conditions was based on the following parameters: discriminatory power and reproducibility. Only PCR reactions generating an average of 10 well-defined and reproducible were further analysed.² A reproducibility test (3 repetitions in different days) was performed using the primer/ annealing temperature combinations that were able to differentiate the reference strains for each bacterial species.

Genomic database: The BioNumerics software was used to analyze and store genomic fingerprint data.

Reporting system: A website containing general information on the pathogens evaluated and hosting the genomic database (BacTrack) was created. www.molecularbacteriology.com.

VII. Results (by objective)

Objective 1 - Develop and validate genotyping techniques for *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, and *Streptococcus suis*.

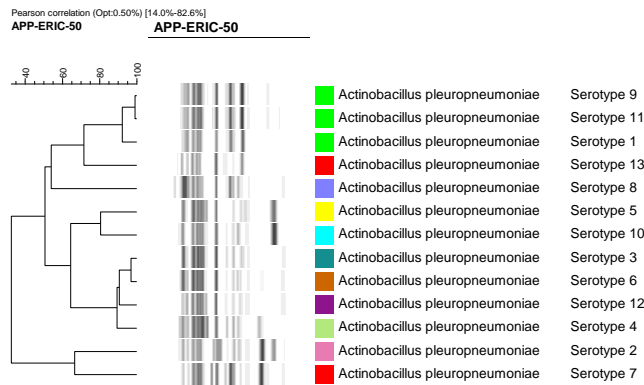
For each of these organisms, 3 sets of primers (REP, ERIC, BOX) and 3 different annealing temperatures were tested (40, 45, and 50°C). The REP primers generated poor fingerprints (less than 5 bands) with inconsistent and non-reproducible results. This primer was not further evaluated. ERIC and BOX primers generated good fingerprints, with more than 10 bands per isolate, and were used for further analysis. Better reproducibility was obtained using the highest annealing temperature of 50°C, and this temperature was utilized for further testing. The discriminatory power of each of individual and combined genotyping techniques was calculated using the Simpson's index of diversity (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php).

Actinobacillus pleuropneumoniae

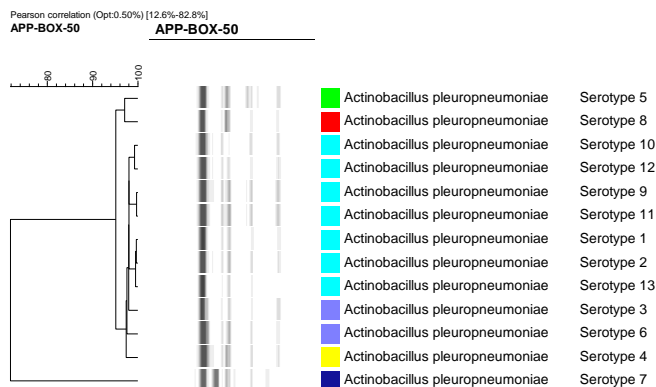
Thirteen references for different serotypes of *Actinobacillus pleuropneumoniae* were used for standardization of the ERIC and BOX techniques. The diversity index for *A. pleuropneumoniae* reference strains was D=0.96 for the ERIC-PCR, D=0.71 for the Box-PCR, and D=0.96 for the combined dendrogram based on both genotyping methods (Figure 1). The ERIC-PCR technique was as discriminatory as the combined fingerprints, and was adopted as the standard method for further characterization of field strains.

Figure 1. *Actinobacillus pleuropneumoniae* Simpson's index of diversity

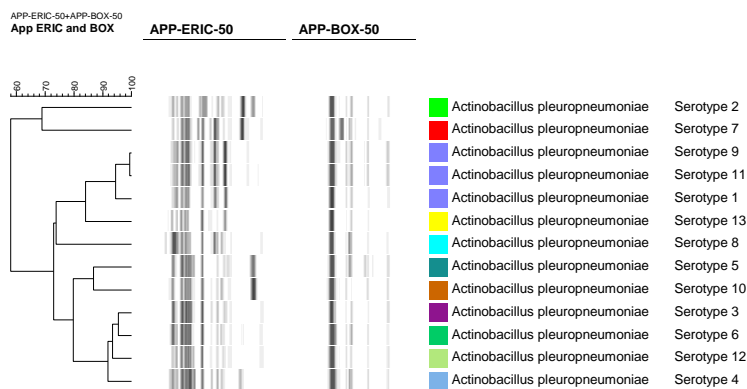
ERIC fingerprints (D= 0.96)



Box fingerprints (D=0.71)



Combined fingerprints (D= 0.96)



Actinobacillus suis

Ten *Actinobacillus suis* field isolates were used to standardize the genotyping technique for this bacterial species. The diversity index was D=0.64 for ERIC-PCR, D=1.00 for the BOX-PCR, and

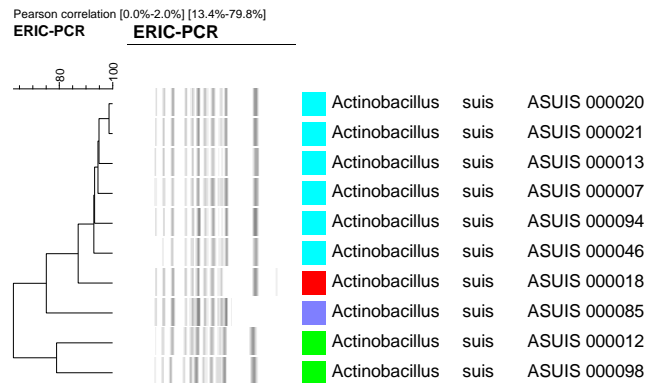
D=1.00 for the combined dendrogram (Figure 2). Characterization of additional field isolates demonstrated that both techniques were needed for better differentiation of *A. suis* strains.

Upon testing of 66 additional field isolates, four fingerprint types were identified by ERIC-PCR and 6 were identified using the BOX-PCR. The Simpson's index of diversity (D) for these techniques changed to 0.17 and 0.49, respectively. Analysis of both fingerprint types in a composite dataset identified 12 fingerprint types (D=0.63). The combined fingerprint types were utilized to define strain groups and evaluate the association between genotype and additional epidemiological parameters.

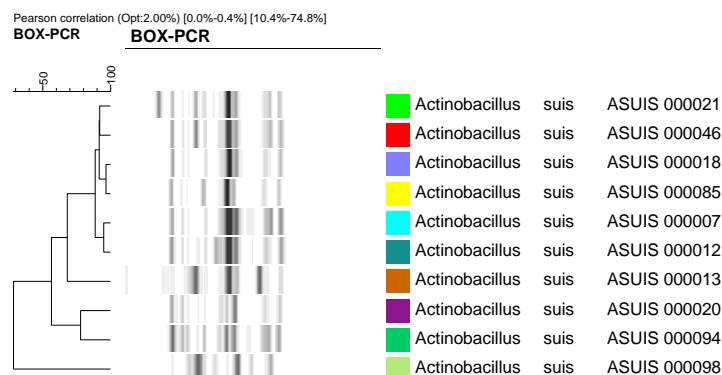
Of the 12 clusters that were obtained using the combined ERIC-PCR and BOX-PCR data, cluster 1 was highly prevalent (59%) among the isolates characterized, followed by clusters 8 (12.1%), 7 (10.6%), and 4 (6.1%). Isolates grouped in cluster 1 were obtained from clinical cases in 2005, 2006, and 2007. This group was identified in 20 different swine herds located in 9 different U.S. states and was isolated from the tonsils (10.3%), lungs (59%), and systemic sites (20.5%) from neonatal (10.3%), nursery (10.3%), finishing (46.2%), and adult (>20 weeks) pigs (18%). The majority (79%) of the herds evaluated had 1 single strain of *A. suis* identified, followed by 2 (18%), and 3 different strains (7%).

Figure 2. *Actinobacillus suis* Simpson's index of diversity

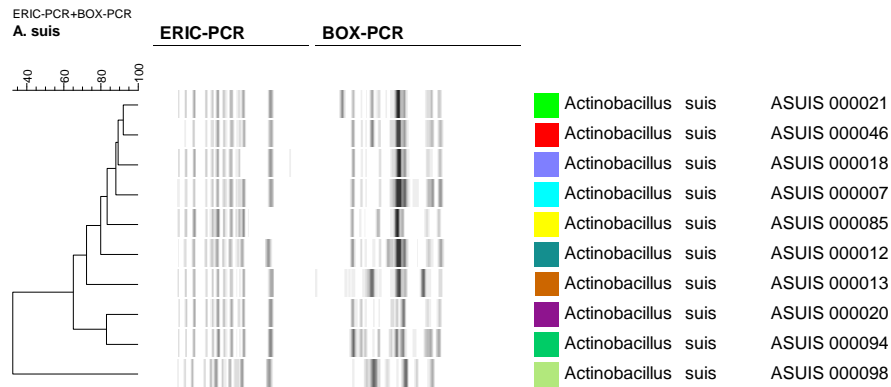
ERIC-PCR fingerprints (D=0.64)



BOX Fingerprints (D=1.00)



Combined fingerprints (D=1.00)

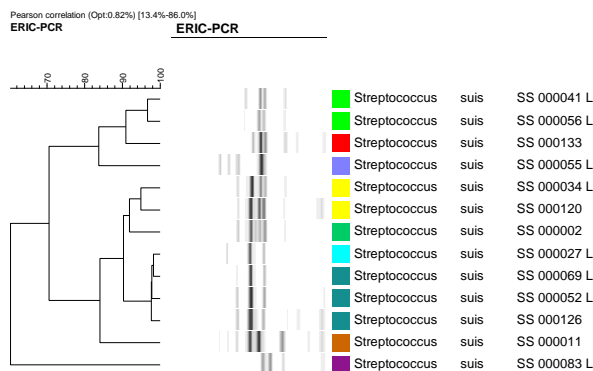


Streptococcus suis

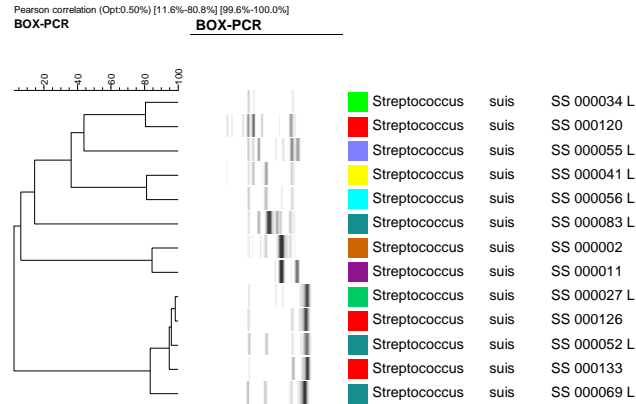
Thirteen *S. suis* field isolates were used to standardize genotyping for this species. These isolates represented different herds and serotypes, and were selected after no differentiation was obtained using the ATCC isolates available for testing. The ERIC-PCR diversity index for *S. suis* was D=0.93, the BOX-PCR index was D=0.92, and the combined dendrogram had a diversity index of D=0.97 (Figure 3). Serotyping was also used to define *S. suis* strains groups, as the dendrogram containing ERIC-PCR, BOX-PCR, and serotyping increased the diversity index to D=0.98.

Figure 3. *Streptococcus suis* Simpson's index of diversity

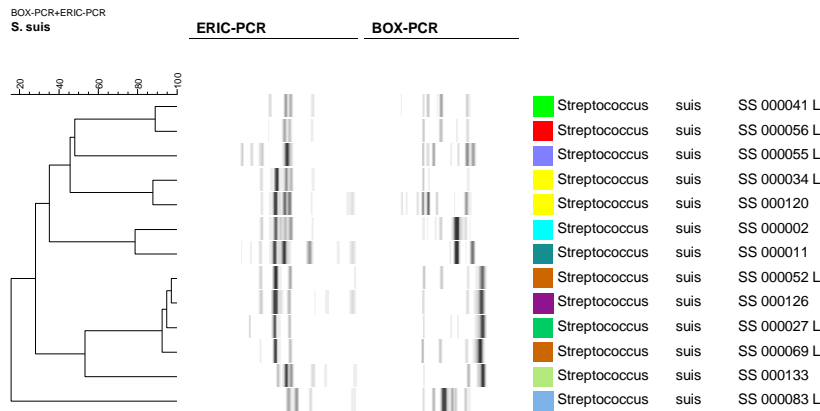
ERIC Fingerprint (D=0.93)



BOX Fingerprint (D=0.92)



Combined fingerprints (D=0.97)



Objective 2 - Create a genomic fingerprint database for *S. suis*, *A. pleuropneumoniae*, *A. suis*, and *H. parasuis*.

Genomic databases were created and are now maintained with routine submissions to the University of Minnesota Veterinary Diagnostic Laboratory. We receive an average of 10 new requests for genotyping per week. All isolates that are genotyped are automatically included in the database and all the clinical and pathological information is also included. Isolates participating in the genomic database were assigned 'Genomic Database IDs', and actual case number are not displayed online.

***Streptococcus suis* genomic database:** The *S. suis* genomic database now contains 141 field isolates from different geographical regions of the United States. It also contains an ATCC human isolate that was recovered from a patient in Canada in 1996 suffering from meningitis. This database is now widely utilized by field veterinarians for selection of autogenous vaccine candidates. We have recently performed 2 studies using the *S. suis* genomic database to evaluate the risk of human *S. suis* infection in the U.S., similarly to what has been observed in China. Although we did not find any isolates on our database that were similar to the highly virulent Chinese strain associated with more than 60 human deaths, we did find at least 3 field isolates that matched the human ATCC strain

based on ERIC-PCR, BOX-PCR, and serotyping. Both studies were presented at the 39th AASV annual meeting, and references are available for consultation:

Publications:

- Clavijo, MJ, Oliveira S. Molecular characterization of *Streptococcus suis* isolates recovered from U.S. swine herds – unraveling the human link. 39th Annual Meeting of the American Association of Swine Veterinarians. San Diego, CA. (Selected for oral presentation in the Students section). **Student award: \$1,500.**
- Santos L, Oliveira S. *Streptococcus suis* infection in the U.S.: serovars, genotypes, and zoonotic potential. Molecular characterization of *Streptococcus suis* isolates recovered from U.S. swine herds – unraveling the human link. 39th Annual Meeting of the American Association of Swine Veterinarians. San Diego, CA. (Selected for oral presentation in the Students section). **Student Award: \$500.**

***Actinobacillus pleuropneumoniae* database:** The *A. pleuropneumoniae* genomic database has been more useful to troubleshoot the biochemical identification of *Actinobacillus* sp. isolates from clinical samples than for selection of isolates to be included in autogenous vaccines. For this pathogen, good protection among isolates from the same serotype (and toxin profile) is usually obtained. The *A. pleuropneumoniae* genomic database now contains 42 isolates, and has been very useful as an identification tool for App-like isolates.

Publications:

Oliveira S., Rossow K, Olsen K, Collins J. (2007) *Actinobacillus* sp. biochemically and phenotypically similar to *Actinobacillus pleuropneumoniae* can be differentiated by genomic fingerprinting, toxin profiling, and sequencing of the 16S rRNA gene. Proceedings of the 50th American Association of Veterinary Laboratory Diagnosticians Annual Meeting, Reno, p. 136. Oral presentation.

***Actinobacillus suis* database:** The *A. suis* database now contains 105 isolates and is also being extensively used by field veterinarian to monitor variability within herds and for selection of vaccine strains. More details on the molecular epidemiology of *A. suis* can be found in this reference:

Publications

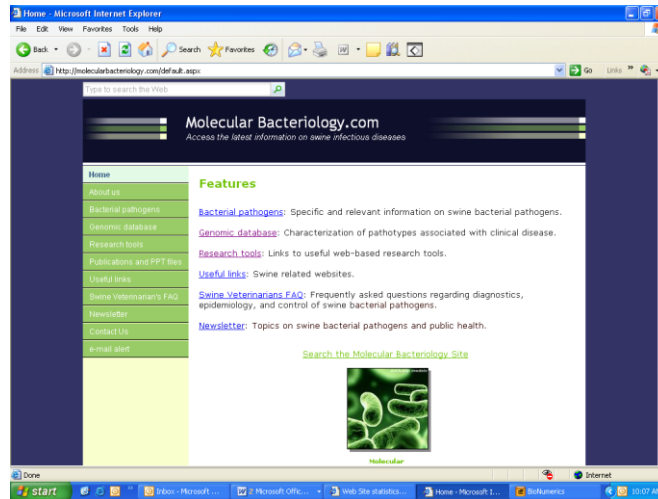
Oliveira S., Tomaszewski J., Collins J. (2007) *Actinobacillus suis* molecular epidemiology. Proceedings of the 50th American Association of Veterinary Laboratory Diagnosticians Annual Meeting, Reno, p. 137. Oral presentation.

Objective 3 - Develop an online identification, reporting, and sharing system to facilitate the use of information stored in the genomic fingerprint database by swine veterinarians and laboratories.

Genotyping is being offered to field veterinarians as a routine test at the University of Minnesota Veterinary Diagnostic Laboratory. We have created a website with general information regarding the main swine bacterial pathogens affecting the U.S. swine industry (Figure 4). This site hosts the genomic database BacTrack at <http://molecularbacteriology.com/database.aspx>, and can be accessed by field veterinarians and other laboratories (Figure 5). Due to the high number of isolates genotyped so far, only the most relevant pathotypes are displayed in the genomic database. This tool has been useful to provide additional information to field veterinarians regarding the virulence potential of specific isolates. We foresee that it will also be very useful to troubleshoot future

outbreaks, including those involving zoonotic pathogens such as *S. suis*. Additionally, our laboratory is constantly monitoring the emergency of new virulent groups using the database.

Figure 4 – The Molecular Bacteriology Laboratory website



www.molecularbacteriology.com

Figure 5 – The BacTrack genomic database webpage.



<http://molecularbacteriology.com/database.aspx>

VIII. Discussion

The objective of this project was to **develop a surveillance system to monitor the genetic variability and molecular epidemiology of swine bacterial pathogens**. A genomic database was created and is being maintained by routine case submissions to the University of Minnesota Veterinary Diagnostic Laboratory. The genomic database can be accessed at the link: <http://molecularbacteriology.com/database.aspx>.

ERIC-PCR genotyping has been used for a number of years as the standard for characterization and selection of *Haemophilus parasuis* strains to be included in autogenous vaccines. The use of this

technique for selection of vaccine strains plays a major role in controlling this costly pathogen in the field. *Haemophilus parasuis* has one of the highest genetic variability among swine bacterial pathogens. Genotyping has been extremely useful to characterize the population structure of this bacterium, identify prevalent groups among North American swine herds, and use this information for disease control. We are now able to do informed decisions regarding the selection of potential vaccine candidates, for example. Considering the value of ERIC-PCR genotyping for *H. parasuis* surveillance and control, we aimed at expanding this concept to other major swine pathogens, namely, *Actinobacillus suis*, *Streptococcus suis*, and *Actinobacillus pleuropneumoniae*.

Genotyping of *A. suis* isolates obtained from U.S. swine herds from different geographical areas revealed that this bacterium is extremely clonal. Contrary to *H. parasuis*, for which more than 90 different strains have been recognized so far, only 12 different strains were identified among *A. suis* isolates characterized by ERIC and BOX-PCR. One of the strain groups identified contains 60% of the isolates tested and was isolated from virtually all production phases and swine herds included in this study. This isolate is now being further evaluated as a potential universal vaccine candidate.

Actinobacillus pleuropneumoniae was successfully genotyped using the techniques tested, with best results and highest discriminatory power being obtained using the ERIC-PCR. The value of *A. pleuropneumoniae* genotyping relies on tracking the introduction of new strains into swine herds. There is no value in using this technique for selection of vaccine candidates, for example, as the toxins produced are the main virulence factors shared by virulent *A. pleuropneumoniae*. Additionally, the App genomic database has been used to troubleshoot the identification of a few *Actinobacillus* sp. isolated from lungs with and without lesions that were biochemically similar to *A. pleuropneumoniae*.

The *Streptococcus suis* database is being widely used by field veterinarians for selection of vaccine strains. This database has been also important to understand the public health risks *S. suis* represents to the North American swine production systems. After a major *S. suis* human outbreak was described in China in 2005, our laboratory used the *S. suis* genomic database to access the similarities between a human *S. suis* isolate recovered from a meningitis case with isolates recovered from swine meningitis in U.S. swine herds. We identified at least 3 *S. suis* isolates that were identical to the human isolate based on ERIC-PCR, BOX-PCR, and serotyping. Although we don't expect the high human mortality observed in China to happen in the United States, mainly due to major genetic differences between U.S. and Chinese *S. suis* strains, we are now prepared to troubleshoot human cases in the U.S. The first human case of *S. suis* meningitis in the U.S was described in 2006, and the human isolate could not be linked to any swine source due to the lack of a surveillance system. That surveillance system is now in place and ready to be used..