OAHN FINAL REPORT

Project #: OAHN-007

Project Title: Culture, antimicrobial susceptibility and molecular typing of *Paenibacillus larvae*, a causative agent of American foulbrood (AFB)

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Executive Summary

American foulbrood (AFB) is the most devastating bacterial brood disease of honey bees (*Apis mellifera*). It is caused by ingestion of spores of *Paenibacillus larvae* by honeybee larvae within 12 to 36 hrs of hatching. Currently, there is very little information available on AFB and its causative agent, *P. larvae,* in Ontario. Limited culture and susceptibility testing for clinical cases has been done in the USDA Bee Research Laboratory. As of December 2015 the USDA laboratory stopped accepting Ontario samples and, therefore, there was a need to have another laboratory available, preferably in Ontario, to provide bacterial culture of *P. larvae* from a variety of samples to the honeybee's industry. More importantly there was a need to monitor susceptibility of *P. larvae* to different antimicrobials used for prevention of AFB as antimicrobial resistance in this pathogen is on the rise. Based on the USDA testing results up to 2012, all Ontario clinical *P. larvae* isolates were susceptible to tetracycline and tylosin. However, there was no susceptibility data available for *P. larvae* from clinically healthy colonies where these drugs are used for AFB prevention. Moreover, there was no information available on ERIC types of *P. larvae* isolates within the Ontario honeybee population even though it has been shown that there is a difference in virulence potential among *P. larvae* of different ERIC types. Among the 4 ERIC types of *P. larvae*, at present, only ERIC I and II appear to be associated with AFB. ERIC I isolates cause typical AFB with larval death at around 12 days of age at the time when cell are already capped whereas ERIC II isolates kill honey bee larvae within 7 days before capping occurs. As a result larvae affected by ERIC II type are frequently removed by nurse bees and typical clinical presentation of AFB is often not present.

Our sample collection from clinically healthy colonies started as scheduled in May of 2016, 2-3 weeks after preventative antimicrobial treatment for AFB. During that time a variety of samples were submitted to and tested in our laboratory for the presence of *P. larvae* including but not limited to brood combs, honey combs, honey, bee wax, pollen, protein patties, hive debris, and bees. Columbia blood agar (BA) and *Paenibacillus larvae* agar (PLA) plates were used for culturing. It was established that for most of the samples using only BA plates and untreated and heat treated samples provided

optimal recovery of *P. larvae* with the exception of honey samples. In honey samples a growth of other *Bacillus* spp. was frequently abundant and as such plating honey on PLA plates ensured optimal recovery of *P. larvae*. The clinical samples were collected during the summer and fall of 2016 as scheduled as well. Unlike culture of samples from clinically healthy colonies the culture of clinical samples yielded good recovery of *P. larvae*. After finishing culturing of samples from clinically healthy colonies in 2016 and no recovery of *P. larvae* our research team made every effort to obtain more samples from clinically healthy colonies through additional OMAFRA recruitment of beekeepers and also by using convenience samples that were submitted to the AHL molecular biology laboratory through the 'Enhanced testing of honey bee health' project. In order to test these samples a real-time PCR was developed for detection of *P. larvae* nucleic acid and used to screen 938 samples. Only 31 samples tested positive by PCR and when culture was attempted from frozen bee samples only 3 *P. larvae* isolates were recovered. As by the end of 2016 our numbers of *P. larvae* isolates was lower than anticipated the project was extended into 2017. For the duration of the project 467 samples were cultured for *P. larvae*, 341 nonclinical samples and 126 clinical samples yielding in total 103 *P. larvae* isolates, 8 from clinically healthy colonies and 95 from AFB affected colonies. It should be noted that the number of isolates does not equal the number of bee yards affected as in some instances multiple isolates of *P. larvae* from the same bee yard were obtained. Despite our team's best efforts in recruitment of more submissions from clinically healthy bee yards the participation stayed low with only 6 additional submissions. As originally proposed Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was successfully used for identification of *P. larvae* as there were already 8 *P. larvae* spectra present in MALDI-TOF Biotyper database. At the end of this part of the project our laboratory was able to provide culture for *P. larvae* using different sample types and also *P. larvae* identification using MALDI-TOF MS. This testing is now available as a part of routine diagnostic service offered by our laboratory and it has been used primarily for honey samples that are being exported to different countries worldwide.

Antimicrobial susceptibility of *P. larvae* isolates was done as originally proposed using tetracycline, and erythromycin as group representatives for tylosin. This initial testing was done following standard operating procedure provided by the USDA Bee Research Laboratory. Based on the initial results all *P. larvae* isolates were susceptible to the drugs tested using disk diffusion (Kirby Bauer) test. However, in the spring of 2017 new CLSI guidelines for susceptibility testing of *P. larvae* also using disk diffusion test became available tilmicosin were included in the testing. Based on these results no resistance to oxytetracycline and tilmicosin were detected.

In order to optimize ERIC and MALDI-TOF MS typing of *P. larvae* a collaboration was established with Dr. Marc Schäffer (Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Germany) as one of the leading experts in MALDI-TOF MS typing of *P. larvae* isolates. His laboratory generously provided well-characterized *P. larvae* reference strains of different ERIC types to be used as controls for ERIC and also for MALDI-TOF typing. Dr. Schäffer also donated 7 additional well characterized field isolates of *P. larvae*. All of the donated isolates were used to create MALDI-TOF MS protein spectra that were added to the Biotyper database in order to develop MALDI-TOF typing. Nucleic acid was extracted from these isolates as well and used for verification of ERIC genotyping (Fig. 1). Initially numerous challenges were

encountered with ERIC genotyping and as a result we focused our efforts on MALDI-TOF typing. To verify some of our MALDI-TOF typing results protein spectra of our field *P. larvae* isolates were sent to Dr. Schäffer's laboratory for confirmation of the results. Confirmation was done blindly and German laboratory confirmed our MALDI-TOF typing results. Based on protein spectra it appears that both ERIC types (ERIC I and ERIC II) are present in Ontario. However, when attempts were made to confirm these results by ERIC genotyping after the procedure was optimized all of the isolates tested were classified as ERIC I type. In order to resolve this issue nucleic acid from 34 field isolates from Ontario will be sent to Germany for ERIC genotyping. We hope to have results back by the end of January 2018.

Objectives

The following objectives were proposed for this project:

1. Culture a variety of samples (honey, brood) for *P. larvae* from clinically healthy colonies and from AFB affected colonies. In total 200 samples from clinically healthy colonies from 8 beekeepers representing different regions within Ontario will be collected in the spring of 2016. Samples from AFB affected colonies across Ontario will be collected throughout the season (summer 2016) as they become available (expecting to collect around 100 samples during that time with multiple samples being collected from the same yard).
2. Use MALDI-TOF MS for identification of *P. larvae*.
3. Perform antimicrobial susceptibility testing of all *P. larvae* isolates using tetracycline and erythromycin disks. These drugs will be used as representatives of the antimicrobial groups of drugs that are approved (oxytetracycline and tylosin).
4. Perform molecular characterization of *P. larvae* isolates using the ERIC method to determine which *P. larvae* ERIC types are present in Ontario.
5. Establish MALDI-TOF typing of *P. larvae* isolates which can be used as a part of routine diagnostic procedure for these isolates since it has been shown that different ERIC types can be detected by MALDI-TOF typing method and ERIC types in *P. larvae*.

Materials and methods

Bacterial isolates

Paenibacillus larvae ATCC 9545 (ERIC I) was purchased from Cederlane. Reference strains of other ERIC types were a generous gift of Dr. Marc Schäffer from Friedrich-Loeffler-Institut, Germany as follows: DSM 7030 (ERIC I), CCUG 48972 (ERIC II), LMG 16252 (ERIC III), and DSM 3615 (ERIC IV). Dr. Schäffer also

donated a few well characterized field strains including ERIC I (BK 100-14, BK 365-13, and BK 385-13) and ERIC II (BK 164-14, BK 199-13A, BK 199-13C, and BK 488-13) representatives.

Sample collection

A variety of samples were collected for *P. larvae* culture from both clinically healthy and AFB affected colonies. Samples from clinically healthy colonies were obtained from several different sources. Initially, 212 samples were collected by Ontario Beekeeper's Association Technology Transfer Program (OBA TTP) staff from 8 beekeepers that were recruited from different geographical areas of Ontario including Beamsville, Niagara, Aylmer, Tara, Norwood, Guelph, Bracebridge and Schomberg. The goal was to collect 25 brood combs per bee yard. The samples were collected in the spring of 2016, 2-3 weeks after preventative antimicrobial treatment for AFB (if used). Out of 212 samples 193 were pieces of brood comb containing a mixture of capped and uncapped cells. The piece of brood comb (20 cm^2) was excised with utility knife, packed in plastic bags, kept cool using ice packs, and delivered to the laboratory for processing. Additional 19 samples collected by OBA TTP included honey, pollen, nectar, wax, hive's bottom debris and protein patties. The second set of samples from clinically healthy colonies was obtained in early September 2016 by randomly selecting a number of beekeepers from OMAFRA database and inviting them to join the project by sending in 4 pieces of brood comb collected as described above and a jar of honey (approximately 100 mL). Only 6 beekeepers were recruited using this approach, among them there was a repeated submission from one of the bee yards that was sampled in the spring. In addition, one beekeeper submitted honey samples (5) only without any brood comb samples. The third and final set of samples from clinically healthy colonies were obtained using convenience samples of bees that were collected over a period of 2015 and 2016 as a part of 'Enhanced monitoring of honey bee health' project spearheaded by OMAFRA. For that project an attempt was made to repeatedly sample bee yards 4 times during the season. In total, 23 yards were sampled in 2015 and 30 yards in 2016. From each yard a cup of bees was collected per hive for up to a total of 10 hives and submitted to AHL molecular biology section for a variety of testing. All clinical samples (i.e., affected brood combs) were collected by Apiary program inspectors from clinically affected colonies during 2016 and 2017 season. In total 126 samples were collected from 20 different yards and submitted to the AHL for *P. larvae* culture.

Sample processing

Brood/honey combs and scales samples. Capped and uncapped cells from brood combs from clinically affected and clinically healthy colonies were swabbed using swabs moistened with phosphate buffered saline and placed into 1.5 ml microcentrifuge vials containing 1 ml of PBS.

Honey samples. Honey was incubated at 41°C for approximately 4 hours to increase viscosity. Twenty milliliters of the warmed honey was pipetted into a 50 mL centrifuge tube and 20 mL of sterile PBS was added. The sample was then shaken to obtain homogenous solution which was centrifuged for 40 min at 6,000 x *g* (7740 rpm). The supernatant was discarded and 1 mL of sterile PBS was added to the pellet and vortexed to re-suspend.

Honey bees samples. 30 honey bees were combined with 20 mL of sterile phosphate buffered saline (PBS) and stomached for 30 seconds. The liquid homogenate was filtered through 1 µM filters and centrifuged at 2200 x *g* (4000 rpm) for 30 minutes. The supernatant was poured off, 1 mL of PBS was added and the pellet was re-suspended by vortexing.

Wax and beehive debris sample. One and a half gram of wax and beehive debris was weighed out into a glass jar. Ten milliliters of xylene was added to it and mixed well. Two milliliters of that solution was pipetted into 6 mL of PBS and vortexed well. A sterile swab was dipped in that solution and plated onto BA and PLA. No pre-treatment of samples was used.

Bacterial culture and identification

After processing samples were plated directly onto blood agar (BA) or heat-treated (5 min at 95°C) and also plated on BA. In addition, majority of samples were directly plated on selective *Paenibacillus larvae* agar (PLA) plates. All plate types were incubated in the presence of 5% CO_2 at 35°C up to 7 days. *P. larvae*-like colonies underwent bacterial identification using Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF MS). Upon identification confirmation bacterial cultures were stored in freezing medium at -70°C until further use.

Antimicrobial susceptibility testing

All *P. larvae* isolates were plated on BA plates and incubated as described above. Bacterial growth was resuspended in phosphate buffered saline (PBS) to achieve turbidity of 1.0 McFarland standard. A cotton swab was used to transfer bacterial suspension to the surface of MYPGP (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate) agar plates. Antimicrobial disks were applied manually within 15 min of plate inoculation. MYPGP plates were incubated at 35°C for 48 hrs before zone of inhibition of bacterial growth were manually measured. As no Clinical Laboratory Standard Institute (CLSI) guidelines were available for *P. larvae* susceptibility testing at the beginning of this project a guideline provided by USDA Bee Research Laboratory, Beltsville, MD was used. Our primary interest was to establish susceptibility to tetracycline in Ontario isolates. In addition, erythromycin was used as the representatives of macrolide drug group instead of tylosin. As of 2017 CLSI guidelines were approved for susceptibility testing of *P. larvae* and tilmicosin was also added.

Nucleic acid extractions, PCR, and ERIC typing

Nucleic acid from *P. larvae* colonies for ERIC typing was extracted using two different kits; Qiagen DNeasy blood and stool kit and Fast DNA Spin Kit for Plant and Animal Tissues. Nucleic acid for 'Enhanced monitoring of honey bee health' project was extracted using MagMAX Pathogen RNA/DNA kit. Enterobacterial repetitive intergenic consensus (ERIC) sequence typing of *P. larvae* was done following procedure as published by Genersh et al. (2006). For *P. larvae* screening of bee samples provided through 'Enhanced monitoring of honey bee health' project the following primers and a probe were used: 16S P. larvae F 5'-ATC ATG GCT CAG GAC GAA C-3', 16S P. larvae R 5'-GCA AGT TAT CCC GGT CTT ACA-3' and 16S P. larvae Probe 5'-AAG TCG AGC GGA CCT TGT GTT TCT-3'-FAM. PCR was done using QuantiFast Multiplex PCR Mastermix with the following cycling parameters: activation -1 cycle at 55°C

for 2 min, denaturation -1 cycle at 95°C for 10 min, PCR-40 cycles of (95°C for 15 sec & 60°C for 1 min), cooling -1 cycle at 40°C for 10 sec.

Results

Recovery of P. larvae from samples submitted and MALDI-TOF MS bacterial identification

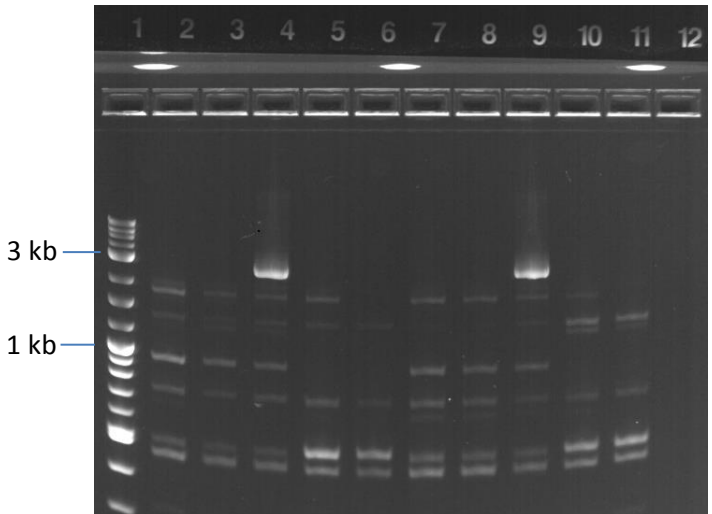
During 2016 and 2017 for the purpose of this project 467 samples were cultured for *P. larvae*, 341 nonclinical samples and 126 clinical samples. There was a very low recovery rate of 2.3% (8/341) of *P. larvae* from samples collected from clinically healthy colonies. In contrast, higher recovery rate of 75.4% (95/126) of *P. larvae* was established for clinical samples. It was determined that *P. larvae* can be easily recovered on BA plates if plating heat-treated and untreated samples without a need for selective medium. PLA selective media was better for recovery of *P. larvae* from honey samples because of occasional overgrowth of BA with *Bacillus* spp. regardless if sample was untreated or heat-treated. MALDI-TOF MS was successfully verified for identification of *P. larvae* as 8 spectra of different strains of *P. larvae* were already present in the Biotyper database. For MALDI-TOF MS identification two different methods of bacterial cell processing were evaluated, direct smear and formic acid extraction and with both methods *P. larvae* identification was achieved for all isolates with MALDI-TOF MS scores >2.0.

Antimicrobial susceptibility testing

Susceptibility testing of all *P. larvae* isolates was done using disk diffusion method and all isolates were tested more than once since CLSI guidelines for susceptibility testing of *P. larvae* became available only in 2017. Regardless of the procedure used for testing all isolates were sensitive to oxytetracycline and tilmicosin.

ERIC genotyping

For ERIC genotyping two different methods of nucleic acid (NA) extraction were compared. Based on our results NA extracted by using Fast DNA Spin Kit for Plant and Animal Tissues provided more consistent results (Fig. 1, lanes 7 to 11). As can be seen in reference strain of ERIC II (Fig. 1, lanes 4 and 9) a distinct band of 2.8 kb is observed which is missing from ERIC I strains. Using this method all isolates tested throughout this project appear to belong to ERIC I group.



MALDI-TOF MS typing

According to Schäffer et al. (2014) MALDI-TOF MS typing can be successfully used to replace ERIC genotyping as a very laborious and hard to reproduce method for typing of *P. larvae* isolates. For that reason spectra of reference strains provided by Dr. Schäffer were added to the MALDI-TOF MS Biotyper database and were used to type *P. larvae* isolates from this study. To confirm some of our results protein spectra were also sent to Dr. Schäffer's laboratory and run through their system database. Our results were a perfect match to their results. Based on MALDI-TOF MS typing of additional field *P. larvae* isolates in this study both ERIC types, ERIC I and ERIC II are present in Ontario honey bee population. It appears that ERIC II is predominant. However, we were not able to confirm these results by ERIC genotyping and at present additional work is required to resolve this issue.

Applications

The following applications resulted from this project:

1. Bacterial culture of *P. larvae* from variety of samples is available to the honeybee industry in Ontario. Producers are now submitting honey samples for culture when required for export purposes.
2. MALDI-TOF MS is used for identification of *P. larvae.* This system allows the same day identification of this pathogen.
3. Additional reference strains of *P. larvae* were added to the MALDI-TOF MS database to increase accuracy of bacterial identification.
4. International collaboration with Dr. Schäffer laboratory has been established.
5. Susceptibility testing is available for all drugs that can be used for preventative treatment of *P. larvae*. This can be used to:
    a. monitor resistance trends in *P. larvae* population,
    b. justify use of a specific drug for prevention of AFB.
6. At present, no resistance to oxytetracycline has been detected in Ontario and as such this drug can safely be used for prevention of AFB.
7. The first database of Ontario's *P. larvae* isolates has been established.
8. ERIC genotyping method has been verified.
9. MALDI-TOF MS typing has also been verified.

Discussion / Suggestions for next steps

American foulbrood (AFB) is the most devastating bacterial disease of honeybee larvae. *Paenibacillus larvae*, a causative agent of AFB, produces spores that can survive in environment for over 35 years. The spores can easily spread from colony to colony by adult honey bees and by different equipment used in beekeeping. Only *P. larvae* spores are infectious and only when ingested by larvae within a few hours

after hatching. Molecular characterization of *P. larvae* isolates using enterobacterial repetitive intergenic consensus (ERIC) showed that there are 4 ERIC types: ERIC I, ERIC II, ERIC III, and ERIC IV. ERIC III and ERIC IV types have not been isolated from AFB cases in decades and only exist as a part of laboratory reference collections. In contrast ERIC I and ERIC II isolates are still associated with AFB. Furthermore, there is a difference in virulence potential of these two types at the larval and colony levels. ERIC I isolates usually kill infected larvae 12 days after infection when cells are already capped. This results in typical clinical presentation of AFB with many cells containing millions of infectious spores contributing to the fast spread of AFB. ERIC II isolates are more virulent killing larvae within 7 days of infection before cells are capped. Nurse bees remove dead larvae reducing the spore loads in hives and as such the spread of AFB in ERIC II infected yards is slower as typical clinical signs are more difficult to detect. ERIC I isolates are widespread whereas it was only recently showed that ERIC II isolates, originally thought to be present in Europe only, are also present in Canada and New Zealand.

There is not much information available about *P. larvae* isolates in Ontario. In the past only clinical cases of AFB were sent to USDA Bee Research Laboratory and susceptibility testing for some of the clinical isolates was done indicating that they are sensitive to oxytetracyline. With the antimicrobial resistance on a raise there is a need to closely monitor susceptibility of *P. larvae* isolates not only for clinical cases but also non-clinical cases where antimicrobials are used for prevention of AFB.

This project was designed to collect and characterize *P. larvae* isolates from Ontario from both clinical and non-clinical cases. Unlike for clinical cases for non-clinical cases there was a very low recovery (3.2%) of *P. larvae* isolates. As only very limited number of bee yards (13) were tested and they were pre-selected it is likely that this number does not accurately represent the situation in the field. With over 6,500 bee yards registered there is a need for a larger study to determine true prevalence of *P. larvae* in yards with no clinical AFB. Among different types of samples used to culture for *P. larvae* it appears that *P. larvae* can be recovered from honey samples as successfully as from any other sample types including brood combs in AFB free yards. As collection and submission of honey samples is less laborious than collection of brood combs it is our recommendation to use honey samples for *P. larvae* culture from non-clinical yards in any future studies. Recovery rate (75.4%) from affected brood combs in clinical cases was relatively high (75.4%) as expected. At the end of the study 103 *P. larvae* isolates were used for susceptibility testing. At present no isolates appear to be resistant to oxytetracycline, a drug predominantly used for preventative treatment of *P. larvae* in Ontario.

As expected MALDI-TOF MS was successfully used for *P. larvae* identification throughout the study. This system was also successfully used for MALDI-TOF MS typing of *P. larvae* isolates. In order to develop the typing system a spectra of reference strains were added to the Biotyper database. MALDI-TOF MS revealed that both ERIC types are present in Ontario. There results were confirmed by Dr. Schäffer's laboratory in Germany. Developing ERIC genotyping method proved to be more challenging with a long optimization process that included two different methods of nucleic acid extraction. When the method has been finally optimized the genotyping results did not correlate well with MALDI-TOF MS typing results. Only ERIC I isolates were detected. As ERIC genotyping is known to be a difficult method to optimize our next step is to send nucleic acid to Dr. Schäffer's laboratory for ERIC genotyping as well.

Until we will get results back we are not able to establish if ERIC II isolates are present in Ontario and also which method can be used in the future for typing of *P. larvae* isolates.

In summary, to the best of our knowledge this is the first project to investigate *P. larvae* population in Ontario. We established the methods for culture and susceptibility testing for this pathogen and also throughout the project collected over 100 isolates to establish the first *P. larvae* database of Ontario isolates. MALDI-TOF MS has been verified as a very convenient and reliable method for *P. larvae* bacterial identification. Resistance to oxytetracycline was not detected. Both typing methods, ERIC genotyping and MALDI-TOF MS typing, work well for characterization of isolates when used on its own. However, when typing results generated by these two methods were compared they did not correlate well. To resolve this issue nucleic acid will be sent to Dr. Schäffer's laboratory in Germany for ERIC genotyping. Once their results will be available we hope to be able to determine which method is more accurate to be used in future studies.

Communications

Publications and/or presentations that resulted from this project:

1. Slavic, D., 2017. American foulbrood (AFB) project update. Ontario Beekeeper Association (OBA) meeting, March 30, Aliston, ON.
2. Slavic, D. 2016. American foulbrood (AFB), what it is and how to spot it. Ontario Bee Journal, July/August.
3. Slavic, D., 2015. American foulbrood project. Ontario Beekeeper Association (OBA) meeting, October 19, Peterborough, ON.

Poster will be also prepared for display at the OAHN annual meeting.