

Monitoring for American Foulbrood Spores from Honey and Bee Samples in Canada

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ABSTRACT

A study of 19 commercial beekeepers was conducted in the Province of Manitoba, Canada. Levels of American foulbrood disease (AFB) were established from these operations by government inspections in the spring of 2002-2004, at which time samples of adult workers were collected from the brood nests of a subset of the colonies. Beekeepers provided representative subsamples of honey across their entire crop. Adult bee and honey samples were pasteurized, plated on modified PLA agar and then the number of spores of *Paenibacillus larvae* subsp. *larvae* that germinated was counted. Antibiotic resistance tests were performed on *P. l. larvae* colonies from each honey sample. Results indicate that number of CFUs/g honey is positively correlated with the number of colonies within a beekeeping operation exhibiting clinical symptoms of AFB, though not a strong predictor. CFUs per adult bee is a better predictor of this parameter, though false negatives in prediction were still encountered. Levels of antibiotic susceptibility to tetracycline and tylosin were established from honey sample isolates leading to the first discovery of a strain of *P. l. larvae* resistant to tetracycline in this region. Results suggest that honey and bee samples have potential as surveillance tools for commercial beekeeping operations in North America.

Keywords: *American foulbrood*, *Paenibacillus larvae larvae*, *honey bee*, *Apis mellifera*, *honey sampling*, *spores*.

INTRODUCTION

American foulbrood (AFB) is a lethal disease of honey bee colonies (*Apis mellifera* L.) caused by the spore-forming, Gram-positive bacterium *Paenibacillus larvae* subsp. *larvae* (Heyndrickx et al. 1996). It is considered to be the most serious disease of bacterial origin that affects honey bees, which are only susceptible to infection by the pathogen at less than 48 h of larval age (Hansen and Brødsgaard 1999). AFB is highly contagious through the spread of the environmentally-stable spores that remain viable in dried larval remains or comb for many years (Matheson and Reid 1992, Shimanuki and Knox 2000). Spores are spread principally through the reuse of contaminated beekeeping equipment, feeding of contaminated honey or pollen to colonies, or through the robbing of dead or disease-weakened colonies by healthy bees.

Because of the severity of this disease, most countries have laws which require mandatory reporting of AFB and the destruction of infected colonies. Over the last 50 years in Canada and the U.S., symptoms of AFB have been suppressed through therapeutic and prophylactic applications of antibiotics, principally oxytetracycline. Although antibiotics kill the vegetative cells, spores remain unaffected. The use of oxytetracycline to control AFB in North America is now in jeopardy with the advent of oxytetracycline-resistant strains throughout the New World (Alippi 1996; Miyagi et al. 2000); resistant strains were first discovered in Canada in the late 1990's (van Westendorp 2001; Tuckey 2002).

An integrated approach to AFB management in Canada is needed to provide a sustainable long-term solution that lessens dependence on antibiotics. With shrinking extension budgets for apicultural professionals, the level of government inspections has steadily decreased. Hence, alternate approaches for the detection of AFB are urgently needed to lessen the dependence on labour-intensive visual detection of disease. One alternative, honey sampling, has been used in several countries to determine the prevalence of AFB spores (Hansen and Rasmussen 1986; Hornitzky and Clark 1991; Steinkraus and Morse 1992; Hornitzky 1999; de Graaf et al. 2001; Fries and Raina 2003; Alippi et al. 2004; Antúnez et al. 2004). Sampling adult workers for AFB has also been investigated, as this technique is more apt to reflect the current disease status of the colony, rather than the status when honey was sampled (Hornitzky 1988, Hornitzky and Karlovskis 1989, Nordström et al. 2002; Lindström and Fries 2005). We chose to determine if enumeration of spores from multiple samples of honey and adult bees could predict AFB levels within commercial honey bee operations in Western Canada. In addition, we used the *P. l. larvae* isolates obtained from honey samples to monitor the extent and spread of oxytetracycline resistance within the province of Manitoba.

MATERIALS AND METHODS

This study was undertaken with the cooperation of beekeepers in the Province of Manitoba, Canada and the Office of the Provincial Apiculturalist. Government inspectors provided data on the incidence of AFB for each beekeeper based on spring assessments and simultaneously obtained adult bee samples. Beekeepers voluntarily provided honey samples.

Sample Collection

Honey Samples: Multiple honey samples were collected from each participating beekeeper. Ten samples were collected from beekeepers with fewer than 350 colonies which were sampled evenly across the harvest of their entire honey crop. Beekeepers with more than 350 colonies, by contrast, collected honey for each 3000 kg increment of honey that was produced, unless a settling tank with less than a 3000 kg capacity was used, in which case a sample was collected each time the tank was emptied.

Bee Samples: In 2004, approximately 30-40 bees were collected into 70% ethanol from each colony inspected by government inspectors. Bees collected from each colony within an inspected apiary were pooled together, such that each sample comprised of a maximum of 25 colonies. Bee samples in 2003 and 2002 represent a large pooled sample taken within a beekeeping operation from inspected colonies, without standardization for numbers of bees sampled per colony.

Microbiological Media

The number of viable *P. l. larvae* spores was estimated using modified PLA (Schuch *et al.* 2001). Modified PLA contained 14.4 g *Bacillus cereus* agar, 10 g trypticase soy broth, 10 g agar, 2.67 g nutrient broth, 3.33 g NaCl, 0.67g Na₂HPO₄ and 1 L nanopure water. Following autoclaving the media was held at 55°C and 25 mL of 50% egg yolk emulsion was added along with filter sterilized aqueous solutions containing 15 mg nalidixic acid, 20 mg pipemidic acid and 16.8 mg amphotericin B.

MYPGP agar (Nordström and Fries 1995) was used for antibiotic susceptibility assays.

Sample Preparation

Honey samples were diluted in sterile phosphate buffered saline (PBS) to enable rapid inoculation of microbiological media. Approximately 300-500 mg of honey was added to 1000 µL PBS in a microcentrifuge tube using a sterile wooden splint. The exact amount of honey delivered was determined gravimetrically.

Adult bee samples were first drained of the 70% ethanol they were collected in and resuspended in 30 mL PBS. The bees in PBS were then transferred into sterile 118 mL Whirl-Pak bags where they were manually crushed to liberate the spores from within their guts. A 1 mL aliquot of each suspension of macerated bees was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 min to separate residual ethanol from the spores. After discarding 900 µL of the supernatant, the pellet was resuspended in 900 µL of PBS.

Tubes containing either the honey solution or centrifuged bee suspension were then pasteurised in dry block heaters for 15 min at 85°C to kill all non spore-forming organisms. With each batch of samples, two tubes containing 1 mL 0.5 McFarland *Escherichia coli* (ATCC 25922) and *P. l. larvae* (NRRL B-3650) suspensions in PBS were also pasteurised. Pasteurisation was determined successful if *E. coli* failed to grow on MYPGP agar (24 h, 35°C) and the *P. l. larvae* strain had comparable viable spores compared to an unpasteurised aliquot.

Enumerating Samples

Viable numbers of spores in each sample were estimated on PLA media using the drop-plate inoculation technique (Herigstad *et al.* 2001). Eight 20 µL drops of honey solution were pipetted onto equal pie-like sections on each plate. Once the drops were dried the plates were incubated at 35°C + 5% CO₂. The number of colony forming units on each plate section was determined at 4 and 7d. Plates with more than 30 colonies growing per drop were uncountable and the original samples were diluted in PBS until between 3 and 30 colonies grew.

Colonies were confirmed as *P. l. larvae* by their morphology and growth rate. Furthermore, one colony from each plate was combined with a drop of 3% hydrogen peroxide on a glass slide to test for catalase production. Catalase production was scored as positive if a colony produced at least one bubble per second. *P. l. larvae* is one of the few spore-forming species from honey bee colonies that grows on PLA that is catalase negative (Haynes 1972).

Antibiotic Susceptibility Assay

All *P. l. larvae* colonies were collected from each PLA enumeration plate after 7 d and transferred onto a single MYPGP plate using a sterile cotton swab. The inoculated MYPGP plates were incubated for 48h at 35°C + 0% CO₂, the culture tested for the absence of catalase production and then harvested into 5 mL of sterile saline to 0.5 McFarlands. These 0.5 McFarland suspensions were used to inoculate MYPGP plates for disc diffusion antibiotic susceptibility assays (NCCLS 2002).

To perform the assay a sterile cotton swab was dipped into the inoculum, rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum. The swab was used to streak-inoculate the dried surface of a 100 x 15 mm MYPGP agar plate. For each honey or bee sample with *P. l. larvae* present three plates were inoculated: two plates each with a 5 µg tetracycline disc (Sensi-Disc, Becton, Dickson and Company, Sparks, MD) and one with a 30 µg tylosin disc (Mastdisc, Mast Diagnostics, Merseyside, UK). To ensure the equivalency among assay results from different days, the susceptibility of three bacterial strains to the two types of discs was determined alongside that of each day's samples. The strains were: *E. coli* (ATCC 25922) and two *P. l. larvae* strains that were alternatively susceptible (NRRL B-3650) or resistant (BLCC-11; isolated in Alberta, Canada) to tetracycline. The zone of inhibition around each disc was determined at 16 to 18 h for *E. coli* and at 48 h for *P. l. larvae*.

RESULTS

Beekeeping Operations and Disease History Ratings

A history of AFB prevalence was constructed for each of 19 beekeepers participating in our honey and adult bee sampling study from data supplied from the Province of Manitoba's government-regulated inspections. Not all beekeeping operations chosen for the study represented typical clinical AFB levels found in the region (<2%), but were instead selected to provide a wide range of clinical symptom history. The size of beekeeping operations ranged from 60 to 3200 colonies per beekeeper with a mean of 790 colonies. In a given year approximately 60 colonies per operation were inspected, ranging from a minimum of 5% of colonies inspected to a maximum of 100 % of colonies, the latter for small operations. During the years 2002-2004, 3 of the 19 beekeepers had no colonies with clinical symptoms of AFB. In sharp contrast, two operations having 100 and 70 colonies, had an average of 65.9% and 60.1% of their colonies infected with AFB over this 3-year period. The remaining operations provided a range of AFB histories in between these extremes.

The beekeeping operations were placed into 5 arbitrary disease history classes, based on the 3-year average percentage of colonies exhibiting AFB symptoms: 0, >0 and <1%, ≥1% and <5%, ≥5% and <20%, and ≥20%.

Honey Samples

Analysis of honey samples submitted in 2003 (*n*=461) and 2004 (*n*=292) shows that regardless of disease history class, we were able to find some level of *P. l. larvae* spores from the majority of samples, with the exception of beekeeping operations having no history of AFB infections (Fig. 1, upper). There is also a close correspondence between years for the proportion of positive samples, however there is no apparent relationship between the number of honey samples positive for *P. l. larvae* and the operations' AFB history.

In contrast, generally higher numbers of CFUs per g honey were found with increasing AFB history categories (Fig. 1, lower). This measure proves more promising as an indication of the number of colonies exhibiting AFB symptoms within beekeeping operations, though it is not highly predictive of the number of colonies with clinical symptoms of AFB (AFB prevalence = $1.95 + 0.01 \text{ CFU/g honey}$; $r^2 = 0.39$; $F = 21.3$; $df = 1, 33$; $P < 0.001$).

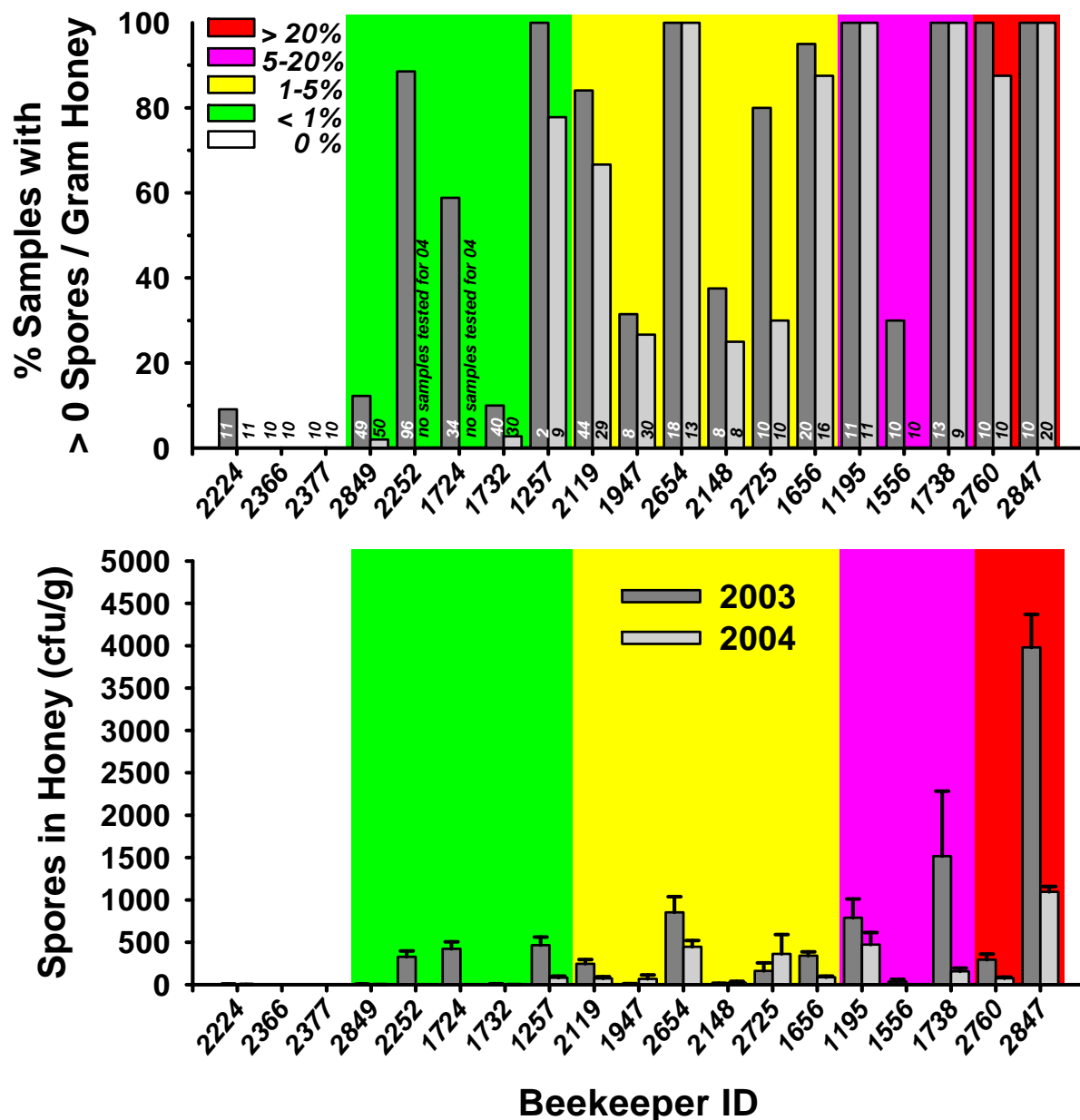


Figure 1. Results of honey sampling from cooperating beekeepers (identity coded by number) from the Province of Manitoba, Canada in 2003 and 2004. Upper graph represents proportion of samples from which any density of *P. l. larvae* could be cultured (positives). Small numbers above beekeeper ID numbers represent the number of samples analyzed. Background colours represent the disease history class to which beekeeping operations belong, based on a 3-year average of proportions of colonies exhibiting visible symptoms of AFB. Lower graph represents actual densities of CFUs/g honey cultured. (CFU=colony forming unit)

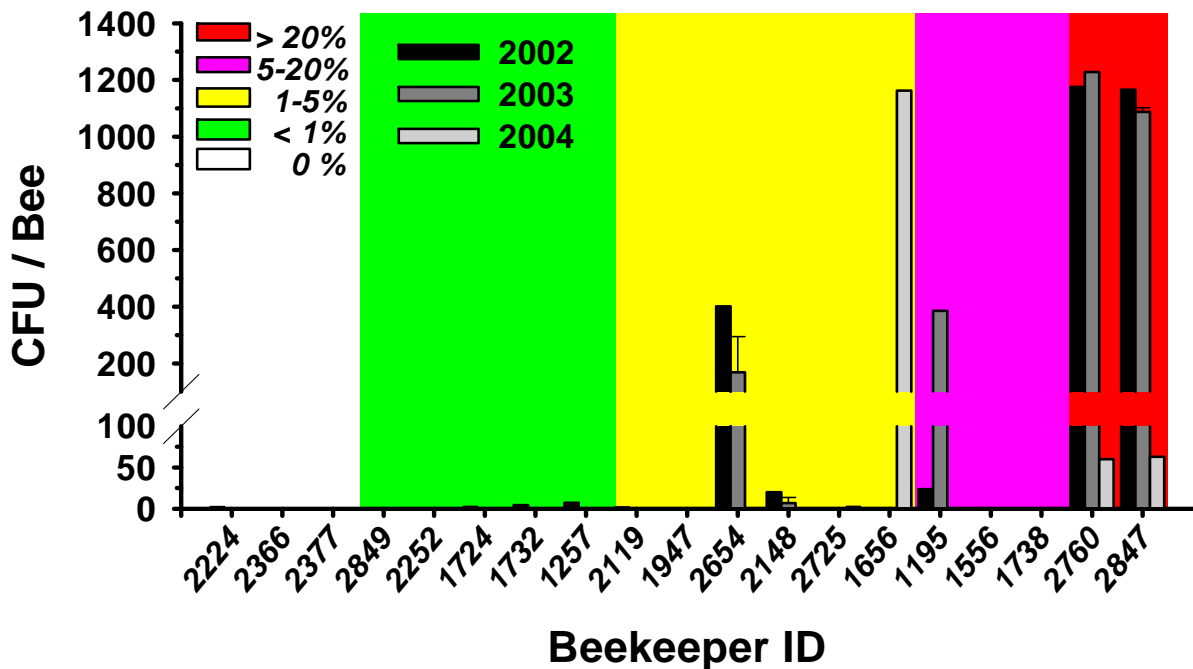


Figure 2. Results of adult bee sampling for cooperating beekeepers from the Province of Manitoba, Canada in 2002, 2003 and 2004. Background colours represent the disease history class to which beekeeping operations belong, based on a 3-year average of proportions of colonies exhibiting visible symptoms of AFB. Samples from 2002 and 2003 are based on large pooled samples of brood nest bees sampled from within a beekeeping operation. Results from 2004 are taken from a composite of standardized apiary-level samples, where each apiary-level sample did not exceed a group of 25 colonies. (CFU=colony forming unit).

Bee Samples

The analysis of bee samples also proved to be a sensitive technique with which to detect *P. l. larvae* spores from beekeeping operations (Fig. 2). In general, greater numbers of CFUs per bee were detected in beekeeping operations which had a greater history of clinical AFB symptoms, though some operations with a high AFB history rank were notably devoid of samples with any *P. l. larvae* growth. Nevertheless, the number of CFUs per bee proved to be highly correlated with the proportion of colonies exhibiting clinical symptoms of AFB during this three year period and a linear regression between these parameters was statistically significant (AFB prevalence = $1.56 + 0.07$ CFU per bee; $r^2 = 0.87$; $F = 329.1$; $df = 1, 48$; $P < 0.001$).

Analysis of 2004 data, in which bee sampling was performed on an apiary basis, permitted comparison of the detection ability of the adult bee sampling method at the apiary level. Out of the total of 58 bee samples processed in this year, 49 were derived from apiaries that contained no visible AFB symptoms. Of these, 44 were negative for AFB, while five were positive. We feel the latter positives provide some indication of the existence of subclinical levels of the disease. The remaining nine apiary samples had some honey bee colonies with visible symptoms of AFB. In five of these *P. l. larvae* could be cultured, but in the remaining four, the pathogen could not be grown from bee samples. Unlike at the level of the operation, no clear relationship existed between the proportion of bee colonies within an apiary with visible symptoms of AFB and the inherent CFU densities cultured from their bee samples.

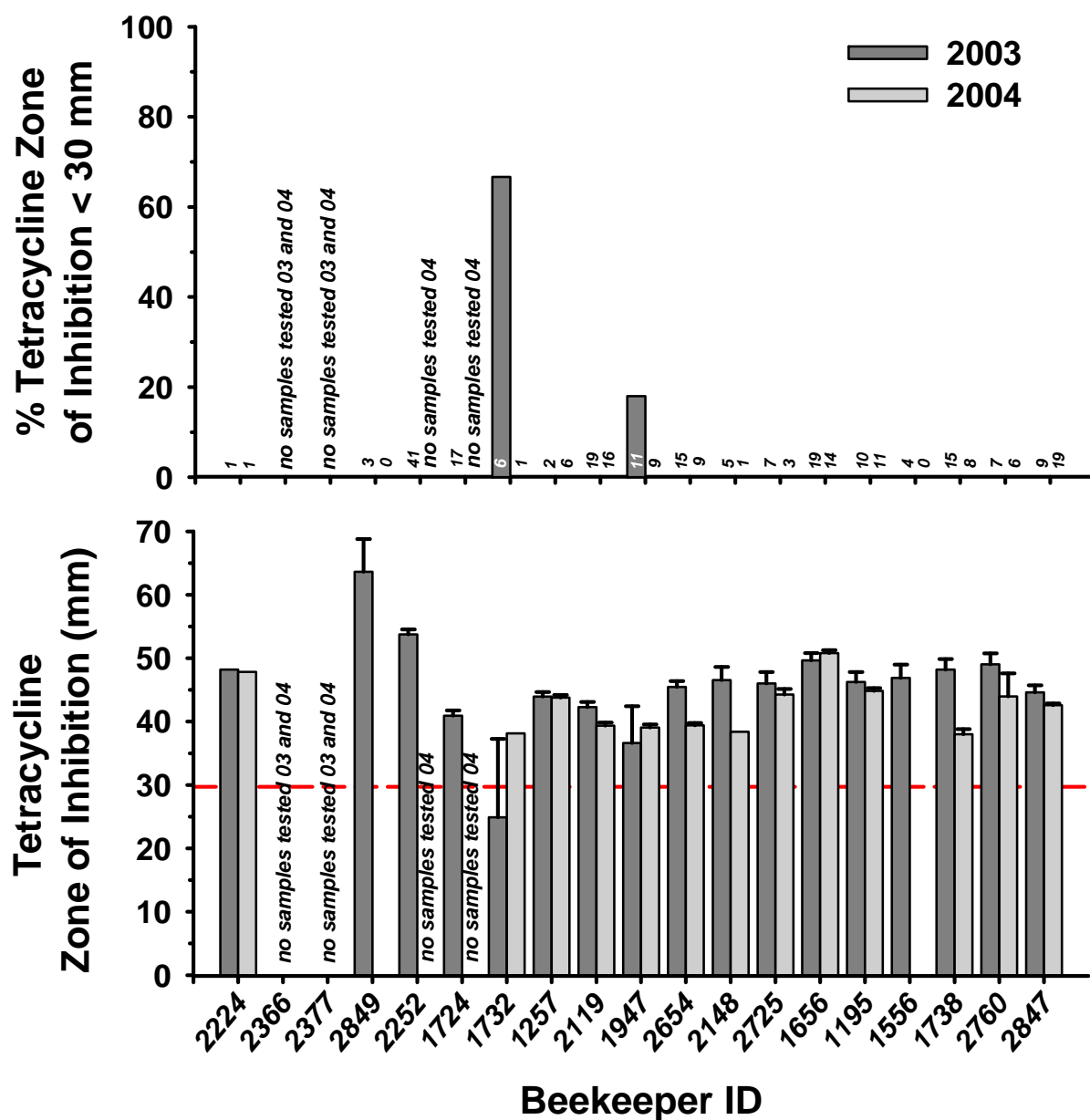


Figure 3. Agar diffusion resistance testing for cooperating beekeepers from the Province of Manitoba, Canada in 2003 and 2004. Resistance testing was performed from *P. l. larvae* isolates, where present and where it could be cultured from honey samples. Upper graph represents proportion of samples from beekeepers that contained *P. l. larvae* strains that were resistant to tetracycline (inhibition zone <30 mm). Small numbers along x-axis represent the number of resistance tests performed. Lower graph shows average zone of inhibition to tetracycline for *P. l. larvae* strains cultured from each cooperating beekeeper's honey samples.

Resistance Testing

From the honey samples in 2003 and 2004, we were successful at performing resistance tests to tetracycline (Fig. 3) and tylosin where *P. l. larvae* could be isolated from individual honey samples. Over all participating beekeepers in Manitoba, the zone of inhibition to tetracycline was 41.7 ± 0.9 (mean \pm SE) mm while to tylosin was 49.2 ± 0.7 mm. These results contrast with an average inhibition zone of 11.1 ± 4.0 mm from a companion study of Alberta

beekeepers, where resistance to oxytetracycline is now widespread. Based on 2003 data, two beekeepers from Manitoba were shown to have *P. l. larvae* isolates that were resistant to tetracycline (zone of inhibition <30 mm, Fig. 3, upper).

DISCUSSION

In this era of ever-decreasing resources for extension apiculture, the use of honey and adult bee samples may be a feasible option for enhanced surveillance of commercial beekeeping operations. We have demonstrated that culturing *P. l. larvae* from honey and bee samples is a reasonably efficient, selective and sensitive way to detect and predict the prevalence of AFB within beekeeping operations in North America, where inherent levels of AFB and management practices differ substantially from many other beekeeping areas of the world. One notable difference is the regular use of antibiotics that mask AFB symptoms leading to inherently higher background levels of spores in bees, honey and beekeeping equipment.

The reliable prediction of the prevalence of AFB in large commercial beekeeping operations using aggregate honey samples faces a number of challenges. One mitigating factor is that colony inspections are typically performed in the spring of the year while honey is collected late in the summer. In the intervening 3-4 months, substantial AFB remediation may take place leading to discrepancies between AFB status of a beekeeping operation at the time the honey samples were taken and the status of colonies during the inspection. In addition, honey samples may also reflect the AFB status of the colony at the time of a particularly strong nectar flow, rather than that of an earlier or later sampling or inspection, adding another potential temporal variation in disease assessments. Moreover, the volume of honey produced may also have an effect on the concentration of spores in honey supers, depending on the size of honey crops annually produced. Finally, the sampling intensity can have a substantial impact on the detection of disease spores. Although our sampling intensity may appear low (ca. 1 sample 3000 kg) this still represents a significant number of samples (50 – 100) for many of our large commercial producers; increased frequency of sampling may not be readily adopted by producers.

Though honey sampling may not be entirely predictive, it still provides a valuable tool to reliably identify beekeeping operations where the risk of AFB is great. We suggest that a threshold level of CFUs in honey may be used to identify a subset of beekeeping operations with the greatest risk of AFB outbreaks. Honey samples appear to provide good reliability to screen out operations in which no apparent AFB risk appears present, which is useful for better targeting extension efforts.

Sampling of honey bees has been previously documented to be a very sensitive technique with which to sample AFB especially for individual colonies (Nordström and Fries 2002, Pernal and Melathopoulos, unpublished data). In Canada, several provinces routinely collect adult honey bee samples for the detection of *Varroa destructor*, *Acarapis woodi* and *Nosema apis*. We have demonstrated that with existing samples of adult bees collected in 70% ethanol, the detection of AFB is possible. These samples are collected at the same time that colonies are inspected for disease and are more highly correlated with inherent disease levels within beekeeping operations as a whole. Although our results indicate that adult bee samples only detected AFB in 5 of 9 of apiaries exhibiting clinical symptoms of the disease in 2004, the rate of positive prediction could be improved by increasing the level of

subsampling. Indeed, false negatives with bee sampling are more likely to occur with composite apiary level samples, especially in situations such as ours in which one colony in a large apiary has only a few diseased cells. Samples of false negatives have also been reported for bee samples in other AFB studies (Goodwin et al. 1996). Although Lindström and Fries (2005) reported no false negatives in their study using adult bee sampling, the inherent proportion of apiaries with clinical symptoms they sampled was very high (54%), the number of colonies per apiary was small, and the disease levels within colonies was relatively high. False positives reported by our study may represent subclinical levels of spores in colonies or variation in the expression of disease due to behavioural resistance factors (Spivak and Reuter 1998, 2001), physiological resistance factors (Rothenbuhler and Thompson 1956, Hoage and Rothenbuhler 1966), or differences in virulence among *P. l. larvae* strains.

Data derived from 2003 Manitoba honey samples was instrumental in the detection of the first cases of antibiotic resistance in this region. The use of honey or bee samples to identify inherent levels of resistance to an antibiotic is an added benefit easily applied to the routine processing of such samples. It can be used to establish baseline resistance data for an antibiotic or trace the invasion of resistant stains within geographical regions. This information can also be applied to enforce quarantines and enhance resistance management strategies.

This study, and a parallel study in the Province of Alberta, is ongoing. We hope to provide more definitive recommendations and analysis of our techniques in the future.

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