OCCURRENCE OF *PAENIBACILLUS LARVAE* SPORES IN HONEY SAMPLES DOMESTIC APIARIES

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Summary

The American foulbrood (AFB) *Histolysis infectiosa perniciosa larvae apium* is an infectious and highly contagious disease of honey bee brood (*Apis mellifera*) and other *Apis* species. Recognition of the AFB in apiaries should be confirmed with laboratory tests. These are tests used in detection of the *Paenibacillus larvae* presence in infected brood. Tests of honey (food stores) for presence of the *Paenibacillus larvae* can detect the infection in bee colonies before the outbreak of the disease and its clinical symptoms. The aim of the research from the years 2005 and 2007 was to do an introductory assessment of the *Paenibacillus larvae* presence in apiaries based on tests of honey samples. A total of 242 honey samples were tested. In microbiological tests, cultivation and microscope methods were employed. In 2005, among 142 samples analysed, 34 were positive for *Paenibacillus larvae*. That was 23% of the total. The same percentage of infected samples was found in 2007 (from 100 tests 23 were positive). Levels of infection varied. The numbers of spores in 1 gram of honey fluctuated from 10 to over 1000.

Keywords: American foulbrood, *Paenibacillus larvae,* spores, diagnostics, honey samples.

INTRODUCTION

The American foulbrood (AFB) is an infectious and highly contagious disease of honey bee larvae. For this reason, controlling AFB is mandatory according to both Polish and EU regulations.

The etiological factor of the disease is a *Paenibacillus larvae* bacterium (Genersh 2006). The bacterium produces a spore form – endospore. Endospores are highly resistant to external factors like high temperature, drying or most chemical disinfectants. The bacterium's ability to survive for many years in adverse conditions (Haseman 1961, Matheson and Reid 1992) and its numerous ways of transmission make fighting the AFB very problematic.

The source of infection is the diseased brood and bee products: honey, pollen, wax and all the apiary equipments in contact with infected colonies. Within an apiary the disease also spreads by bee-movement. Movement such as drifting or robbing of dead or disease-weakened colonies by healthy bees. Transporting hives to distant honey flow, results in contamination of areas which had been disease-free. Detection of AFB in the field is usually based on the appearance of typical clinical symptoms in the brood. By the time of this relatively late diagnosis extensive and uncontrollable spreading of the disease between bee colonies may have taken place. The ability of the infection to go undetected for long periods of time even years aids in its spread.

The outbreak of AFB in different bee colonies may vary. Many factors are

involved. They include the number of endospores in bees, and the genetic predisposition of bee populations to recognise and remove diseased larvae. The bee colonies with good hygienic behaviour are able to quickly eliminate infected larvae and consequently eradicate large parts of a potential infection source. The progress of the disease in colonies with good hygiene is much slower.

It is possible to detect the presence of the *Paenibacillus larvae* infection in bee colonies even before the appearance of AFB clinical symptoms. This is done by laboratory analysis of bee products (Ohe 1997, Hansen and Brodsgaard 1999). The Hansen method of *Paenibacillus larvae* detection in honey (1984), has led to a world wide application in estimating AFB presence in bee colonies (Hansen and Rasmussen 1986, Shimanuki and Knox 1988, Hornittzky and Clark 1991, Ritter 1992, Steinkraus and Morse 1992, Hornitzky 1999, de Graaf et al. 2001, Fries and Raina, 2003, Alippi et al. 2004). As a result of research a correlation has been found between the level of *Paenibacillus larvae* infection of honey and occurrence of AFB symptoms in honey bees (de Graaf et. al. 2001, Ritter 2003, Prenal and Melathopoulous 2006). The detection method allows for early preventative treatment against development and spread of the disease. The detection method helps to reduce the costs and workload of fighting AFB.

In recent years in Poland cases of AFB have been reported only sporadically. According to the data from the Veterinary Hygiene Institute there were 45 cases reported in Poland in 2005 and 23 cases in 2006. So far there has been no research into the existence of AFB in Polish apiaries. The one published work on the subject looks only at the Małopolska province (Lipiński et al. 2007). Therefore the true health of bee colonies as far as the AFB outbreak and *Paenibacillus larvae* infection are concerned, is unknown.

The aim of the research was to assess the extent of the *Paenibacillus larvae* infection in honey samples; and consequently of bee colonies. The aim of the research is also to legitimize country-wide, long term, regular monitoring of the epizootic situation when it comes to AFB. It becomes essential to explain the role of AFB in the Colony Collapse Disorder syndrome. The method used in the research allows detection of sub-clinical infections. In many apiaries, the outbreak of AFB (clinical symptoms) can be avoided by early preventative action.

MATERIAL AND METHODS

The research was done in the Bee Disease Laboratory, of the Departament of Parasitology, in the National Veterinary Research Institute in 2005 and 2007.

In 2005 the test material were honey samples taken by 142 beekeepers from the Śląskie province. The samples from all colonies were taken from sealed honey comb cells close to the brood nest. They were collected in one container and constituted a collective sample from an apiary.

In 2007 the research was based on 100 honey samples taken from commercial honey containers. The honey came from apiaries located in 12 provinces. The 12 provinces were: Dolnośląskie, Mazowieckie, Warmińsko-Mazurskie, Świętokrzyskie, Opolskie, Wielkopolskie, Małopolskie, Pomorskie, Lubuskie, Podlaskie, Lubelskie and Kujawsko-Pomorskie. The number of samples available for the research from particular provinces was varied. The honey samples were taken from labelled containers. This way it was known which honey varieties they came from: nectar – multifloral, buckwheat, heather, acacia,

lime, rape; nectar and honeydew; honeydew.

The microbiological research was based on detection of the presence or absence of bacterial colonies typical for *Paenibacillus larvae* (cultivation method). Microscope and biochemical confirmatory tests were used. From each sample, 5 g of honey were diluted with 5 ml of sterile distilled water. They were then mixed together. Next, the beaker was covered with foil and incubated for 10 minutes at 90°C (initial incubation). Using a sterile pipette, 0.4 ml was deposited on a Petri dish with a Columbia sheep blood agar medium. For each test sample, 3 dishes were inoculated by spreading the honey solution using sterile Drygalski spatulas. Following the inoculation, the Petri dishes were incubated in 37 ± 1°C. On the 3rd and 6th day the dishes were then observed and the bacterial colonies identified based on their morphology. *Paenibacillus larvae* grows both as small (diameter 1-2mm) and larger (3-4mm) greyish-white, non-transparent, flat colonies, usually with uneven, milky edges. The characteristic colonies were marked on the Petri dishes and then counted. From a single colony recognised as morphologically typical for *Paenibacillus larvae,* a sample was then taken for microscope testing (nigrosine dyeing – Plageman test) and for a catalase test (with 3% hydrogen peroxide).

The result of each test was given as the average number of colony-forming units/dish (CFU). The average number of colonies grown on the dishes was the basis to determine the extent of the infection of bee colonies. In the study of honey samples with *Paenibacillus larvae* spores, 3 degrees of infection were assumed:

1) No bacterial colonies developed – level of infection 0

2) From 1-45 CFUs per dish – low level of infection

3) Over 45 CFUs per dish – high level of infection

RESULTS

Among 142 samples tested in 2005 the presence of *Paenibacillus larvae* was detected in 34 samples (23% of the total number). The same percentage of infected samples was shown in 2007 (in 100 samples 23 were positive). The level of infection of individual samples varied widely. For 1g of honey, the content of bacteria was calculated to be from 10 to over 1000. In 2005 from 33 infected samples, 70% qualified as low level of infection and 30% as high level of infection. In 2007 a low level of infection was noted in 80% of positive samples (Table 1). The average infection levels in both years were similar.

Table 1.

Prevalence of *P. larvae* spores in honey samples.

Year	Number of samples	Number and (%) of positive samples	Average number of CFU/dish (range)	Positive samples (%)	
				Low infection level < 45 CFU	High infection level > 45 CFU
2005	142	33(23)	87.8 (1-185)	69.7	30.3
2007	100	23(23)	94.6 (1 – 292)	78.3	21.7
Total	**242**	**56(23)**	**91.2 (1 – 292)**	**74.0**	**26.0**

CFU - colony forming unit.

In the research the highest number of infected samples were from the Mazowieckie province. Almost half of the samples from this area contained *Paenibacillus larvae* spores, but the average infection level in these samples was low. However, in the samples from Warmińsko-Mazurskie and Dolnośląskie provinces, both the high percentage of infected samples and high levels of infection were present. In samples from the Lubelskie province, despite a high number of samples tested, no presence of *Paenibacillus larvae* was detected (Table 2).

Table 2.

Prevalence of *P. larvae* spores in honey samples from different provinces.

Year	Province	Number of samples	Positive samples (%)	Average number of CFU/dish
2005	śląskie	142	23.0	87.8
2007	mazowieckie	8	62.5	9.4.
	warmińsko-mazurskie	25	48.0	137.5
	dolnośląskie	11	36.4	151.0
	świętokrzyskie	3	33.3	2.0
	małopolskie	14	7.1	9.0
	opolskie	10	0	0
	wielkopolskie	1	0	0
	pomorskie	1	0	0
	lubuskie	1	0	0
	podlaskie	1	0	0
	lubelskie	24	0	0
	kujawsko-pomorskie	4	0	0

CFU - colony forming unit.

The results of testing for presence of spores in known honey varieties showed the highest percentage of positive samples in heather honey (half of the samples tested positive). Also in buckwheat honey the percentage was significantly higher than in other varieties (Table 3).

Table 3.

Prevalence of *P. larvae* spores in different honey samples.

Honey type	Variety	Number of samples	Positive samples (%)
Nectar	Heather	8	50.0
	Buckwheat	11	27.3
	Acacia	7	14.3
	Lime	11	9.0
	Multifloral	11	9.0
Honeydew	Coniferous	9	11.1

DISCUSSION

In recent years in Poland, cases of the AFB have been reported only sporadically which may suggest that incidences of the disease remain undisclosed by beekeepers. As a result the disease is not controlled and can spread unrestricted. Results of the research show that the percentage of apiaries infected with *Paenibacillus larvae* is relatively high. This indicates a large spread and frequent occurrence of the disease in the country. The data gained imply that the cases of the American foulbrood disease are more common than the number of official notifications to the Veterinary Inspector may suggest. Lipiński et al. (2007), having tested 251 samples from 9 districts of the Małopolskie region proved the existence of *P. larvae* in 51 samples tested (20.3%). This percentage is similar to the one found in this work. Among the positive samples found in Lipiński's research, a low level of infection was present in 22 samples (8.8% of total samples tested and 43.1% of positive samples). A high level of infection was found in 29 samples (11.5% of total tested and 56.9% of positive samples). In our research a high level of infection was detected in the lower number of positive samples (20-30%).

We also discovered a lower percentage of *P. larvae*-infected-samples among the ones from the Małopolskie province compared to what Lipiński found. However, this results from a much lower number of samples from this area (14), that were tested in our research. Because of varied numbers of samples from different regions, the results of this analysis cannot be related to the actual spread of the infection in the regions.

Findings regarding the occurrence of *Paenibacillus larvae* infections in foreign apiaries are highly varied. In Uruguay (Antunez et al. 2004) the first clinical case of the AFB was found in 2000. In 2001 and 2002 during tests of 101 honey samples from 19 provinces *P. larvae* was found in 52 samples (51.5%). In Brazil Schuch et al. (2001) used a method similar to the Antunez's and tested 137 samples of imported honey and 300 samples of Brazilian honey. They detected *Paenibacillus larvae* in 24 samples of the imported honey. According to Ritter's research (2003) only 2% of honey samples from German apiaries contained *Paenibacillus larvae* spores. The spores were detected in 98% of the 700 samples of honey imported to Germany from non-EU countries and in 62% (from 200 tested) of honey samples from the EU. Similar research was done in Germany in 1993-1996 by Von der Ohe (1997). Following tests of 2099 samples of German and foreign honeys, the research showed a presence of *P. larvae* in only 7% of the German honeys while all the foreign samples (from Argentina, Greece, Iran, Russia and the US) had very high contents of *P. larvae* spores. In Belgium, AFB had been noted only sporadically (23 cases between 1997-1999). In the research from 2000, Graaf et al. (2001) showed 11% of the 1328 Belgium honey samples tested to be infected with *Paenibacillus larvae*.

CONCLUSION

— The data gained shows the considerable extent of the *Paenibacillus larvae* presence in apiaries. This indicates the risk for development of the American foulbrood disease in the apiaries infected and those in their vicinity.

— The test results point at a high advisability of systematic monitoring of the AFB in Poland. Such an undertaking is necessary to assess the epizootic situation of the AFB in apiaries. Systematic monitoring will

allow for improvement of existing conditions. It can lower the risk of the disease developing, without needing to destroy bee colonies.

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WYSTĘPOWANIE SPOR *PAENIBACILLUS LARVAE* W PRÓBKACH MIODU POCHODZĄCYCH Z KRAJOWYCH PASIEK

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S t r e s z c z e n i e

Zgnilec amerykański pszczół (hystolysis infectiosa perinciosa larvae) jest zakaźną i wysoce zaraźliwą chorobą czerwia pszczoły miodnej (*Apis mellifera*) i innych gatunków z rodzaju *Apis*. Rozpoznanie zgnilca amerykańskiego w pasiekach powinno być potwierdzone badaniami laboratoryjnymi, polegającymi na stwierdzeniu obecności bakterii *Paenibacillus larvae* w chorym czerwiu.

Badanie miodu (zapasów pokarmu) w kierunku obecności *Paenibacillus larvae* pozwala na wykrycie zakażenia rodzin pszczelich bakteriami jeszcze przed rozwojem choroby, któremu towarzyszy wystąpienie objawów klinicznych. Badania te mogą być podstawą epizootycznej oceny występowania bakterii zgnilca amerykańskiego w rodzinach pszczelich i umożliwiają pszczelarzom wykonanie odpowiednio wcześniej zabiegów profilaktycznych, ograniczających rozprzestrzenianie się bakterii i rozwój choroby.

Celem badań wykonanych w roku 2005 i 2007, była wstępna ocena występowania bakterii *Paenibacillus larvae* w pasiekach na podstawie badania prób miodu. W roku 2005 do badań pobrano próbki miodu bezpośrednio z rodzin pszczelich, ze 142 pasiek zlokalizowanych na terenie województwa śląskiego. W roku 2007 do badań pobrano próbki, z miodu znajdującego się w handlu. Miód ten pochodził z pasiek zlokalizowanych na terenie 12 województw: dolnośląskiego, mazowieckiego, warmińsko-mazurskiego, świętokrzyskiego, opolskiego, wielkopolskiego, małopolskiego, pomorskiego, lubuskiego, podlaskie, lubelskiego i kujawsko-pomorskiego. Łącznie przebadano 242 próbki miodu.

Badania mikrobiologiczne przeprowadzono metodą hodowlaną i mikroskopową. Średnia liczba kolonii wyhodowanych na płytkach była podstawą do określenia stopnia zakażenia rodziny. Na 142 próbki przebadane w roku 2005, obecność bakterie *Paenibacillus larvae* wykryto w 34 próbkach, co stanowiło 23 % ogólnej ich liczby. Taki sam procent zakażonych prób wykryto w roku 2007 (na 100 prób - 23 próby były pozytywne). Poziom zakażenia poszczególnych próbek był bardzo zróżnicowany i mieścił się w zakresie od 10 do powyżej 1000 bakterii w 1 gramie miodu. W roku 2005 spośród 33 zakażonych prób miodu w około 70% prób stwierdzono niskie zakażenie sporami *P. larvae*, a w 30% zakażenie wysokie. W roku 2007 zakażenie na niskim poziomie stwierdzono w 80% prób pozytywnych. Przy porównaniu zależności miedzy odmianą miodu, a poziomem zakażenia okazało się, że najwięcej bakterii zawierały miody wrzosowe i gryczane, co jest zgodne z przebiegiem rozwoju choroby w ciągu sezonu.

Uzyskane wyniki świadczą o istotnym rozprzestrzenieniu bakterii *Paenibacillus larvae* w pasiekach, a tym samym wskazują na ryzyko rozwoju zgnilca amerykańskiego w ich obrębie bądź w obrębie sąsiadujących pasiek. Uzyskane wyniki wskazują jednocześnie na konieczność podjęcia w kraju systematycznych badań monitoringowych w celu poprawy obecnej sytuacji i zmniejszenia ryzyka rozwoju tej choroby, bez konieczności likwidacji rodzin.

Słowa kluczowe: zgnilec amerykański, *Paenibacillus larvae,* spory, diagnostyka, próbki miodu.