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SCREENING OF *PAENIBACILLUS LARVAE* SPORES IN APIARIES FROM EASTERN POLAND. NATIONWIDE SURVEY. PART I

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Abstract

Screening of the prevalence of *Paenibacillus larvae* spores in honey bee colonies in apiaries from 162 districts, belonging to nine provinces was carried out during 2009–2011. The honey samples were examined by the use of a culture method. Based on the number of CFUs grown on Columbia sheep blood agar medium, the level of infection and probability of American foulbrood outbreak was estimated. Altogether, 6,510 pooled honey samples from 32,550 bee colonies located in 2,294 apiaries were collected. *P. larvae* was identified in 45% of the surveyed apiaries. The widest distribution of *P. larvae* was found in the Małopolskie province. Culture–positive honey samples were obtained for 71% of the apiaries and in a half of them, the level of spores was high. In the Warmińsko-Mazurskie province, the presence of the bacterium was detected in 58% of the apiaries. In the remaining provinces, from 26% to 47% of the apiaries were contaminated with *P. larvae* spores.

Key words: *Apis mellifera*, *Paenibacillus larvae*, spores, American foulbrood, honey, Poland.

American foulbrood (AFB) is a highly contagious and lethal disease affecting larval stage of *Apis mellifera* and other *Apis* species. The causative agent of the disease is the spore-forming bacterium *Paenibacillus larvae* (3). Bee larvae are infected by food containing spores of the bacteria. In the body of the host, *P. larvae* multiplies and is able to produce millions of spores in each infected bee larva. Spores are unusually resistant to the environmental factors and remain viable in dried larvae, food stores (honey, pollen), and combs for many years (23). So far, four genotypes (ERIC I to ERIC IV) of *P. larvae* species were identified. These genotypes differ in their phenotype and virulence on the larva level. The larvae infected with ERIC I, die within 12-14 d. Other genotypes cause a larval death within 6-7 d. However, all genotypes of the bacteria cause similar pathologies of the infected larvae (2, 3).

Diagnosis of AFB, based on the characteristic clinical symptoms, can be confirmed by the identification of *P. larvae* in the samples of brood collected from a suspected colony (16). Stores of food (honey, sugar syrup, pollen), wax, adult worker bees, and winter hive debris can also be used when looking for *P. larvae* spores in honey bee colonies. Bacteriological or molecular methods used to study the materials are sufficient to detect a sub-clinical level of infection (4, 5, 10, 12, 20, 24, 25). The culturing method of honey samples developed by Hansen (7), and its numerous modifications, has been often used for the national AFB monitoring programme (1, 6, 7, 9, 11, 14, 18, 19, 22, 23,). Based on the correlation between number of *P. larvae* spores in the honey and occurrence of visible symptoms of the disease, the risk of AFB outbreak in honeybee colonies can be determined with a high probability. The early detection of *P. larvae* allows preventive measures to be taken and helps to reduce the spread of the disease.

These days, beekeepers rarely report a suspected occurrence of the disease to the veterinary inspectors. On the other hand, beekeepers have reported an increased mortality of bee colonies in recent years. American foulbrood can be one of the causes of an abnormal health status of a honeybee colony.

In 2009, a five-year screening was started to estimate the prevalence of *P. larvae* and the risk of the development of AFB in national apiaries. The studies are based on identification of the pathogenic agent in honey samples collected from bee colonies during the beekeeping season.

Material and Methods

The study was carried out in nine Polish provinces: Lubelskie, Podkarpackie, Świętokrzyskie (in

2009), Mazowieckie, Podlaskie, Warmińsko-Mazurskie (in 2010), Małopolskie, Śląskie, and Opolskie (in 2011). Apiaries designated for the survey were based on *P. larvae* prevalence revealed in preliminary investigations (19). Within each of the surveyed provinces about 10% of all registered apiaries were selected for the study. The selected apiaries were distributed proportionately across the whole area of the provinces (all districts).

While being selected, the proportion of apiaries with different numbers of colonies was taken into account (consistent with the structure of the apiaries in Poland). Apiaries from groups counting from 6 to 10, from 11 to 20, and from 21 to 50 bee colonies had a similar percentage of apiaries selected for the study – 25%, 26%, and 27%, respectively. The percentage of apiaries with 51-80 colonies was significantly lower (7%). The least numerous group (2.5%), were the apiaries, which had over 80 colonies. The study included 2,294 apiaries, in which the total number of bee colonies was approximately 66,700.

Honey samples were collected during the beekeeping season (from June to September). The samples came from cells from the top of the brood combs. One sample consisted of approximately 50 g of honey collected from five randomly selected bee colonies per every 10 bee colonies in the apiary. This allowed to examine at least 50% of the colonies from each apiary. The number of samples collected from each apiary was conditioned by its numerical force (from 1 to 10 samples). A total of 6,510 samples were collected from 32,550 bee colonies.

Laboratory tests were conducted with the use of the van der Ohe method (15). Isolation of *P. larvae* from honey samples was conducted by culturing bacteria on Columbia sheep-blood agar media supplemented with nalidixic acid (25 mg/L). Cultures were incubated for 6 d at 36 ±1°C, under aerobic conditions with 5%-10% CO_2. *P. larvae* isolates (including four genotypes) were identified by colony and bacteria morphology (Gram staining), and they were identified biochemically (catalase test). The number of *P. larvae* colonies was counted. Based on an average number of colony forming units (CFUs) obtained from cultures on three Petri dishes, the level of contamination of the sample was determined:

- 0 CFUs/Petri dish (0 spores/g honey) – negative result of laboratory test, *P. larvae* spores were not detected in the sample;
- up to 45 CFUs/Petri dish– positive result of the laboratory test, *P. larvae* spores were detected in the sample, the level of the sample contamination was classified as low;
- above 45 CFUs/Petri dish – positive result of laboratory test, *P. larvae* spores were detected in the sample, the level of the sample contamination was classified as high.

Based on the level of infection obtained for individual samples, epizootic status of *P. larvae* infection in each apiary was determined according to the following criteria:

- category 0 – no *P. larvae* infection in the apiary;
- category I – low content of spores in the apiary (positive result of the laboratory test for at least 1 sample but a high level of *P. larvae* spores was not detected in any of the samples);
- category II – high content of spores in the apiary (positive result of the laboratory test with a high level of *P. larvae* spores for at least 1 sample).

The chi-square test was used to compare the proportion of positive samples collected from apiaries of nine provinces.

Results

P. larvae was cultured from 35.6% of the total examined honey samples. Results obtained for the individual provinces were significantly different since the samples contaminated with *P. larvae* ranged from 18.9% to 64.3% (Table 1). The least amount of culture-positive honey samples (below 25%) was detected in Podlaskie, Mazowieckie, Opolskie, and Śląskie provinces. From four other provinces, the infected samples were from 30% to 45%. In the Małopolskie province, there were nearly twice as many samples containing *P. larvae* spores than in any of the other provinces.

During the 3-year study, the presence of *P. larvae* spores were detected in 1,029 (44.9%) out of 2,294 examined apiaries. A high level of infection (classified as category II) of honey bee colonies was found in 17.2% of the apiaries. The prevalence of *P. larvae* in the apiaries and the level of contamination of the honeybee colonies with spores, differed significantly between the provinces and districts (Figs 1, 2, 3).

Lubelskie province. *P. larvae* spores were present in 43.9% out of 271 monitored apiaries located in 20 districts. There were no bacteria detected in the honey samples collected from the three districts (Pulawy, Luków, and Parczew). In the remaining 17 districts, the percentage of infected apiaries ranged from 23% to 73%. Widespread infection was observed in seven districts (Tomaszów Lubelski, Opole Lubelskie, Radzyń Podlaski, Kraśnik, Wlodawa, Ryki, Bilgoraj), in which at least half of the apiaries had positive samples. In the Bilgoraj and Ryki districts, infection was found in approximately 70% of the surveyed apiaries. Bee colonies were highly contaminated with *P. larvae* (category II) in 5% to 36% of the apiaries from 14 of the districts, and in 60% of the apiaries from the Wlodawa district (Figs 1, 2, 3).

Świętokrzyskie province. The study included 230 apiaries from 13 districts. Samples collected from 37.4% apiaries had positive laboratory test results. There were no infected apiaries recorded in two districts (Busko Zdrój and Sandomierz). In 10 districts, there was 10% to 46% occurrence of the *P. larvae* in apiaries, but in the Kielce district the percentage reached nearly 60. Apiaries classified as category II had from 10% to 26% occurrence of *P. larvae*.

Table 1
Rate of contamination with *P. larvae* spores of the honey samples collected from the nine provinces.

Province	Number of examined samples	Proportion of culture-negative samples (%)	Proportion of culture-positive samples (%)		
			low level of infection	high level of infection	Total*
Lubelskie	808	68.3	20.3	11.4	31.7 c
Podkarpackie	739	62.5	23.3	14.2	37.5 c
Świętokrzyskie	678	68.9	1.6	12.5	31.1 c
Mazowieckie	972	78.1	18.1	3.8	21.9 ab
Podlaskie	377	81.1	14.9	4.0	18.9 a
Warmińsko-Mazurskie	948	56.5	34.3	9.2	43.5 d
Małopolskie	1,006	35.7	37.9	26.4	64.3 e
Śląskie	695	75.4	16.3	8.3	24.6 b
Opolskie	287	78.4	15.3	6.3	21.6 ab
Total	6,510	64.4	23.9	11.7	35.6

*chi-square test. The different letters (a, b, c, d, e) indicate significant differences between the means (P<0.05).

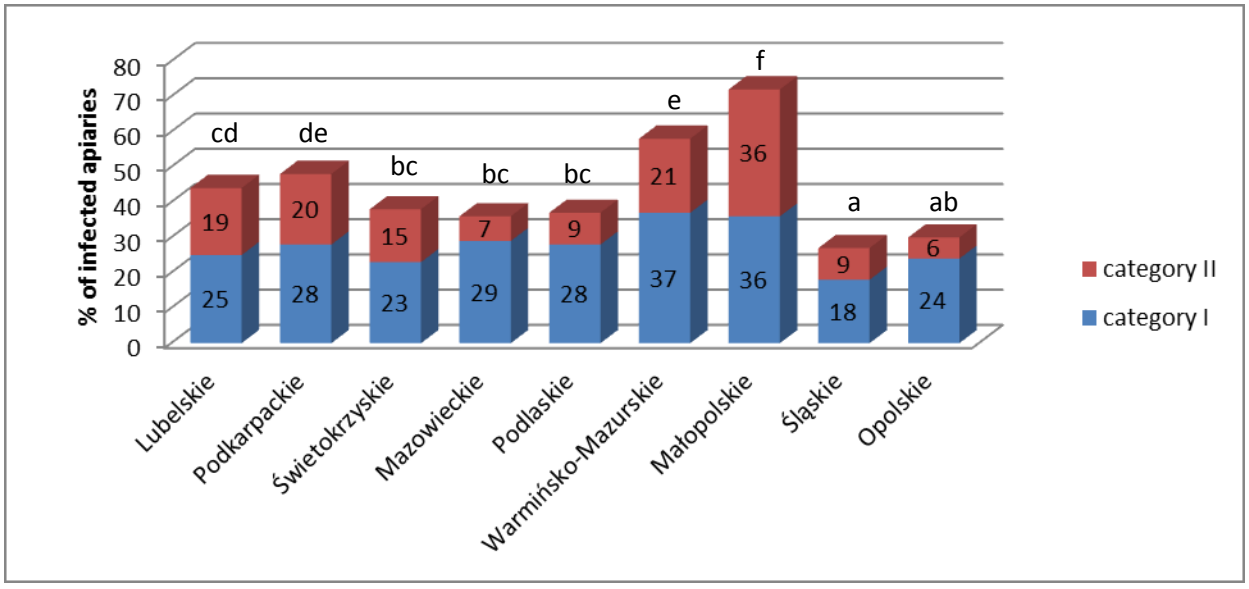


Fig. 1. Proportion of the apiaries with a low level of *P. larvae* infection (classified as category I) and with a high level of *P. larvae* infection (classified as category II), in the nine surveyed provinces.
*chi-square test - the different letters (a, b, c, d, e, f) indicate significant differences between the means (P<0.05).

Podparpackie province. Among of 251 apiaries selected for monitoring from Podkarpackie province (including 19 districts), *P. larvae* was isolated from 50% of the apiaries. Distribution of the infected apiaries varied; in 10 districts – from 10% to 30% of the apiaries were infected. In the remaining nine districts, from 50% to 90% of the apiaries were infected. A high level of spores was detected in 20% of the apiaries. However, in Sanok and Przemyśl, where *P. larvae* occurred in 90% of the apiaries, there was a high level of infection in 46.7% and 56.4% of the apiaries, respectively.

Mazowieckie province. The survey was conducted in 302 apiaries located in 35 districts. The culture-positive honey samples were found in 36% of the apiaries. In the Białobrzegi and Gostynin districts, *P. larvae* was not detected. In 11 districts, the rate of infected apiaries was below 30%, in other 11 districts it ranged from 33% to 48%. A wide prevalence of American foulbrood bacteria (in 50% to 79% of the apiaries) was also noted in 11 provinces. However, apiaries with an intensive degree of infection did not exceed 7.3%. In the majority of apiaries (located in 18 districts), only a low amount of *P. larvae* spores (classified as category I) was reported. Przasnysz and Węgrów had the highest rate (33.3%) of the apiaries classified as category II.

Podlaskie province. In this region, monitoring included 127 apiaries. Honey samples containing bacterial spores were found in 36.3% of them. In 12 districts, contaminated apiaries ranged from 10% to 50%. In Kolno and Mońki, *P. larvae* was isolated from 75% of the apiaries. Intense infection was noted in approximately 9% of the apiaries located only in six

districts. A particularly large number of apiaries (75%) were classified as category II in the Mońki district.

Warmińsko-Mazurskie province. In the Warmińsko-Mazurskie province, the incidence of *P. larvae* was high and amounted to 58% from 231 examined apiaries. There were infected apiaries in all 18 surveyed districts, and in only five districts, the percentage of apiaries with *P. larvae* was less than 50. In the remaining 13 districts, the bacteria were present in 50%-77% of the apiaries. In six districts (Bartoszyce, Węgorzewo, Braniewo, Lidzbark Warminski, Olsztyn, Ostróda), the infection occurred in more than 70% of the apiaries. A high level of *P. larvae* spores was detected in 13 districts, in 21% of the apiaries. In the Iława and Ostróda districts, the category II apiaries were significantly more than in the remaining districts, and they amounted 53.8% and 57.1%, respectively.

Małopolskie province. Results of the survey performed in nearly 400 apiaries revealed a wide distribution of *P. larvae.* In these regions, as much as 71.3% of the apiaries were infected, and in half of them, the bee colonies had a high level of bacteria. From the 16 districts of the Małopolskie province, only one (Dąbrowa Tarnowska) had no *P. larvae* detected whilst in 13 districts, the contaminated apiaries ranged from 50% to 97%. The highest prevalence of *P. larvae* (97%) was observed in three districts (Tarnów, Myślenice, Nowy Sącz). Most of these areas also had the category II apiaries (42.4, 55.2, and 65.4%, respectively).

Śląskie province. In the Śląskie province, 378 apiaries were examined. Culture-positive honey samples were found in 26.5% of these apiaries. The apiaries classified as category II amounted to 8.7%. In 15 out of 16 monitored districts, *P. larvae* was identified. In six districts, however, the number of infected apiaries did not exceed 15%. The most widespread occurrence of the bacteria was in the apiaries of Bielsko-Biała, Kłobuck, and Myszków districts.

Opolskie province. In two districts, no *P. larvae* was detected from any of the honey samples. In the remaining nine districts, bacteria were isolated, on average, from 30% of the apiaries. In five districts, the proportion of infected apiaries exceeded 30%, while in four (Prudnik, Nysa, Opole, Brzeg) the proportion of infected apiaries ranged from 50% to 60%. Only four districts had apiaries with a high number of identified spores (from 5% to 20%).

Discussion

The results from 2009 to 2011 revealed that *P. larvae* is a widespread microorganism. The bacterium was isolated from 45% of the sampled apiaries. The presence of spores may not only lead to the outbreak of the disease in the apiaries, but also poses a threat to infection-free apiaries located nearby. Particularly dangerous is the proximity of apiaries with a diagnosed high risk of the AFB outbreak (17% of all examined). With regard to the geographical location and administrative division, the situation is very diverse. Based on the studies carried out till now, two large regions were found particularly at risk of the AFB infection. The two regions are located at the opposite ends of the country. These at-risk regions are: a wide area in southern and south-eastern Poland, and a smaller one in northern Poland.

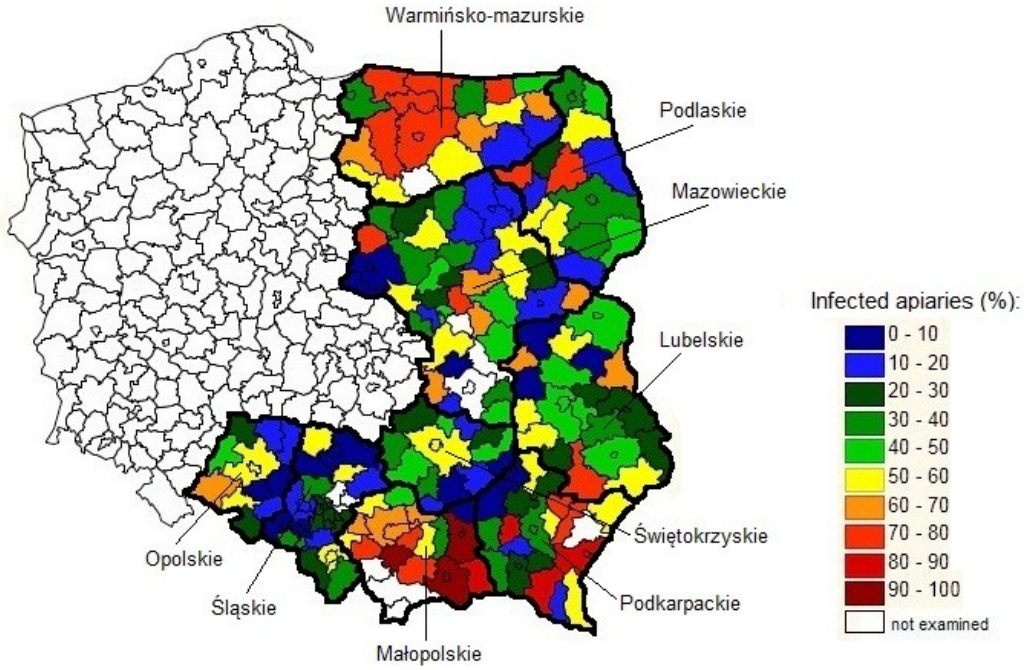


Fig. 2. Proportion of the apiaries contaminated with *P. larvae* in the individual districts of the nine monitored provinces.

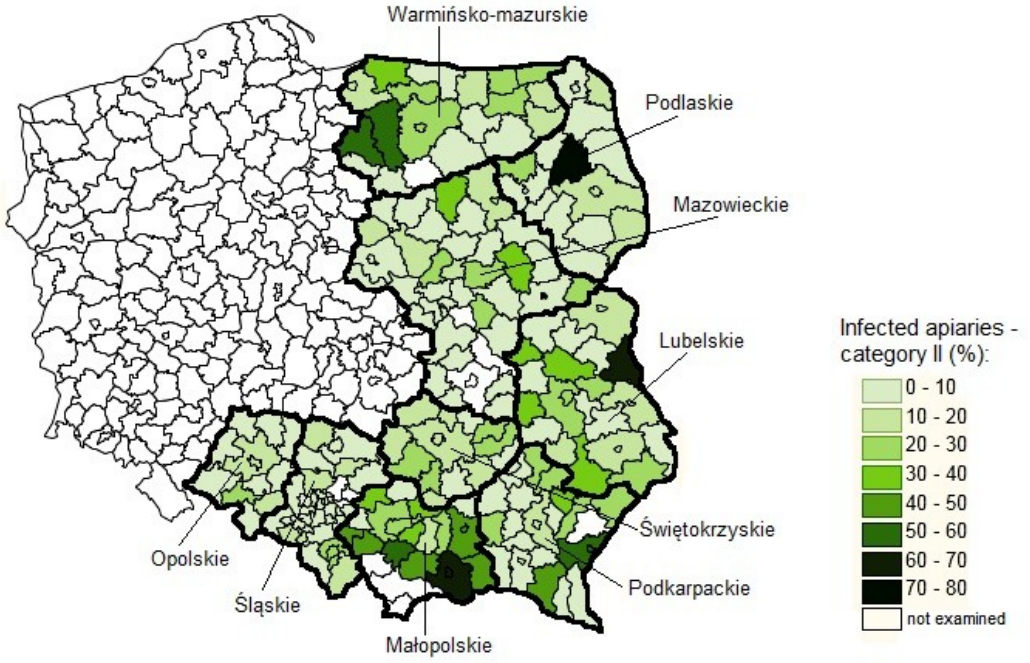


Fig. 3. Proportion of the apiaries with a high degree of *P. larvae* infection (classified as category II) in the individual districts of the nine monitored provinces.

In the south, the area at risk extends along three neighboring provinces; the worst situation is in the Małopolskie province. In the province, the total percentage of both infected apiaries and those with a high level of infection is the largest among the nine administrative provinces surveyed till now. In the Małopolskie province, *P. larvae* bacteria were detected in samples from 71.3% of the apiaries, and half of the apiaries probably poses a high risk of occurrence of the AFB clinical symptoms. In terms of epidemiology, the risk of disease outbreak concerning nearly the whole area of the province is very serious. In as many as 13 (out of 16) districts, infected apiaries make up between 50% and 100% of the total number. In 10 districts, at least one third of the apiaries have colonies with a high number of spores. The apiaries located in the 10 districts are particularly threatened by a disease outbreak. These districts are: Wieliczka (31% of the apiaries classified as category II), Proszowice (33%), Olkusz (36%), Gorlice and Oświęcim (each 41%), Tarnów (42%), and Limanowa and Wadowice (each 43%). The worst situation is in the Myślenice and Nowy Sącz districts, where almost all the surveyed apiaries were infected (around 96%) and a high number of spores was found in more than half of them (55% and 65%, respectively).

In apiaries located in the south-eastern area of Poland, administratively belonging to the Podkarpackie province, spores were detected, in total, in 48% of the apiaries. A high percentage of infected apiaries (above 50) was noticed over a large area, that is, in about half of the districts located in the province. In the surveyed apiaries, 20% were classified as having a high risk of the disease outbreak. However, unlike the Małopolskie province, most of these high-risk apiaries were located in two districts only: Sanok and Przemyśl.

In apiaries situated in the Lubelskie province, the total percentage of infected apiaries was 44%. The ratio of apiaries with category II contamination was 18.5%. The overall results are similar to those obtained for the Podkarpackie province but an unfavourable epizootic situation was found in only seven of the 20 districts. As far as epidemiology is concerned, it is important to note that some of these districts adjoin those of the Podkarpackie province in which the proportion of infected apiaries was also high. The areas particularly being at risk in the Lubelskie province (in terms of the highest ratio of apiaries with a high number of spores) are regions around the districts of Bilgoraj, Opole Lubelskie, Ryki, and Wlodawa.

The another region particularly at risk of AFB outbreak (high ratio of infected apiaries in total, high percentage of category II apiaries, as well as, a widespread distribution of infected apiaries) is located in the north of the country; in the Warmińsko-Mazurskie province. *P. larvae* spores were found in nearly 60% of the apiaries in this region. As in the Małopolskie province, the spore distribution covers a wide area. A high proportion of infected apiaries (between 50% and 77%) was found in 13 (out of all 18) districts. In comparison to the Małopolskie province, the number of contaminated category II apiaries is lower by 14 percentage points. This is the second highest (after the Małopolskie) ratio of apiaries of this category found so far. However, in case of Warmia and Mazury, the apiaries are located in a smaller number of districts, when compared to the Małopolskie province. The greatest risk (between 30% and 50% of apiaries

classified as category II) was found in the following districts: Ilawa Ostróda, Braniewo, Goldap, Lidzbark Warmiński, and Olsztyn.

In the Małopolskie, Podkarpackie, and Lubelskie provinces, the number of registered apiaries is the largest in Poland and varies between 4,800 and 5,600 (21). When analysing the results of the high percentage of infected apiaries, and especially of apiaries classified as category II (30%, 20%, and 19%, respectively), it can be assumed that the risk of the spread of the infection in these areas is much higher than in other provinces. Colony density is the largest in that area when compared to the rest of the country, and it must be also taken into consideration. The number of colonies per square kilometer in the Małopolskie province is 7.2, in the Podkarpackie– 6.7, and in the Lubelskie 6.4. In the Warmińsko-Mazurskie province, the number of registered bee colonies and colony density is also very high in the country (21). The structure of the apiaries, though, is different. There are about half as many apiaries, but they are larger. An average apiary is made up of approximately 44 colonies. Due to these differences, the number of infected apiaries in the Warmińsko-Mazurskie area will be lower compared to the three provinces mentioned above (despite the high percentage of infected apiaries in the Warmińsko-Mazurskie area).

Among the Podlaskie, Mazowieckie, and Świętokrzyskie provinces, the AFB epizootic situation proved to be very similar. The presence of *P. larvae* spores was detected in about 1/3 of the apiaries located within each of them. Due to low levels of infection, the majority of the apiaries were classified as category I. From among all the surveyed apiaries, these accounted for between 22% and 28%, while 7%-15% of the apiaries had a high number of spores. Investigations carried out so far, indicate that the Opolskie and Śląskie provinces have the lowest spread of AFB bacteria, so these provinces are at the lowest risk of the disease. The ratio of infected apiaries amounted to 30% and 26% for the Opolskie and Śląskie provinces, respectively. The percentage of category II apiaries does not exceed 10%.

Until 2009, the AFB epizootic situation in Poland was not evaluated on such a large scale. Previously, the published data concerning the presence of *P. larvae* in Polish apiaries was random, and it presented the results of studies based on a relatively small number of samples or concerning only a small area (11, 19). In the preliminary studies conducted between 2005 and 2007 by Pohorecka and Bober (19), among the 142 samples of honey collected from apiaries and 100 samples of packed honey, the proportion of infected material amounted to 23%. This amount is lower by 13 percentage points when compared to the current results. In 2005, the proportion of infected apiaries in the Śląskie province amounted to 23%. The result was similar to the result obtained in 2011 – 26% of infected apiaries.

Epizootic situation of the apiaries of the Małopolskie province obtained by Lipiński in 2007 (11) differs from those presented in the paper. Of the honey samples collected by Lipinski from 251 apiaries in nine districts, 20.3% were positive. Infected apiaries constituted the same proportion because from each of the apiaries only one pooled sample of honey was collected, whereas, the presence of spores was found in 64% of 1,006 samples collected from 397 apiaries in 16 districts, in 2011. This accounted for 71% of the apiaries. The significant difference in the results of the studies carried out after a 4-year interval may be the result of increasing the detection threshold by making the number of collected pooled samples dependent on the size of the apiary (one pooled sample for every 10 colonies), and/or a continuous spread of the infection, and an increase in the number of clinical cases of the disease during this period.

In Poland, there are about 45 thousand registered apiaries. In 2009, AFB clinical symptoms were recorded in 80 of the registered apiaries, in 2010 – in 96, and in 2011 – in 147. Therefore, over the past 3 years, the disease has been found each year in 0.2%-0.3% of all apiaries in the country. The three-year screening has showed that, on average, 17% of the apiaries are at high risk of AFB development due to the high level of infection with *P. larvae* spores. When comparing the results, it can be assumed that a significant proportion of AFB cases is not recognised or reported by the owners of the apiaries.

Out of the 323 AFB outbreaks reported by the General Veterinary Inspectorate in 2009–2011, most outbreaks were located in the provinces of Małopolskie, Podkarpackie, Śląskie, and Warmińsko-Mazurskie (disease was recorded in 72, 56, 45, and 16 places, respectively). In other provinces during that period, either single cases of the disease or none at all (Łódzkie and Lubuskie provinces) were registered. These statistics are in the agreement with the results of our screening. The highest percentage of infected apiaries was found in the provinces where the veterinary services recorded the largest number of AFB clinical cases. A wide distribution of *P. larvae* spores may be the result of an approximately threefold increase in the risk of infection in the neighboring apiaries (6). The Śląskie province is an exception, as based on the screening it would be considered to be at the lowest risk of AFB development.

The comparison of the epizootic situation of American foulbrood in different regions of Europe or the world is difficult, primarily because of the lack of research standards (including sampling standards). The investigation was carried out with the use of a varied number of honey samples collected directly from bee colonies. Honey after extraction and stored in containers of varying volume, as well as, samples of packed honey were also used. German studies are the most corresponding to Polish studies. In Germany, the disease development is recorded in between 100 and 400 apiaries per year from a total of 100 thousand apiaries. This gives a ratio of 0.1% to 0.4%, which is similar to the ratios recorded in Poland. However, the results of German monitoring differ significantly. Out of 5,000 samples of food stores collected from colonies between 1993 and 1996, and in 2003, the presence of AFB spores was found in 7%, of which 5% were samples with low

levels of infection. In areas at higher risk, the proportion of infected samples amounted to 23% (14, 16). According to von der Ohe (13), in Germany the 10% apiaries are infected with *P. larvae*. In the study, the total amount of infected samples was almost 36% (24% with cat. I contamination, 12% – with cat. II). In the Małopolskie province, which had the largest number of AFB clinical cases, the share was as large as 64%. The differences between the percentage of infected samples and apiaries in Poland and in Germany (while a similar number of officially registered cases of the disease are observed) may support the hypothesis that many infections were not being reported. The discrepancies may also result from the fact that for German studies, usually only one pooled sample was collected from each apiary. For a similar reason, the results of the studies conducted in the Belgian apiaries turned out to be very surprising to the Belgium researchers. In Belgium (with about 10 thousand apiaries), only about eight cases of the disease were officially recorded each year while studies revealed the presence of *P. larvae* spores in 11% of honey samples (6).

Despite the fact that the presence of spores is necessary for AFB outbreak, other factors may also modify the occurrence and progress of the disease. Varied resistance of individual larvae or colonies (*e.g.* resistance associated with hygienic behaviour of colonies) or differences in the pathogenicity or virulence of different *P. larvae* strains may be decisive (8, 2). The data obtained during the screening should be considered as an indicator of high risk regions of the country, which require the control and prevention activities.

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