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Kısa Bildiri ***Short Communication***

Prevalence of American Foulbrood and *Paenibacillus Larvae* Genotypes in Bulgaria

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Abstract

This study aimed to analyse the prevalence of American foulbrood and *Paenibacillus larvae* genotypes in Bulgaria. For this purpose, data concerning American foulbrood outbreaks were used. Also, available data on the number of destroyed bee families covering a twenty-five-year period (1989 - 2013) was collected from the register of Bulgarian Food Safety Agency. In addition, *Paenibacillus larvae* genotypes in 15 apiaries were established by rep - PCR with BOXA1R and MBOREP1 primers. Results showed broad incidence of the disease, particularly at the beginning of the analysed period, with a tendency to reduction of affected apiaries and bee families at the end of the period. A statistically significant prevalence of American foulbrood in South Bulgaria compared to the northern regions was observed. Genotyping of *Paenibacillus larvae* revealed two genotypes: ab and AB, with the preponderance of the more virulent AB in South Bulgaria. The obtained results showed the need for conducting regular mandatory screening of bee colonies for early diagnostics of the disease and the implementation of relevant measures for American foulbrood prevention and control.

Introduction

American foulbrood is the most debilitating bacterial disease of larvae of honeybees *Apis mellifera* and *Apis cerana*, inflicting serious economic losses on apiculture worldwide (Mill et al., 2014). The etiological agent, *Paenibacillus larvae* (*P. larvae*) exists in two forms: vegetative rods, which are non-infectious, and spores - the only capable ones of causing the infection (Genersch, 2010). Whereas vegetative cells perish quickly under the influence of physical and chemical factors, spores are extremely resistant to the adverse environmental conditions (Pernal and Melathopoulos, 2006). Furthermore, *P. larvae* spores in scales of larvae remains are viable for several decades (Genersch, 2010). Hence, the prolonged storage of the pathogen in different apiary sources. Moreover, this is a prerequisite for an easy transmission of the spores within the colony and between colonies (Fries and Camazine, 2001). American foulbrood spreads by horizontal and vertical mechanisms (Lindström et al., 2008). While, the disease is transmitted mainly horizontally, the vertical mode, occurring in the process of bee swarming, is the pathogen's way of survival and remaining in the bees' nests (Fries et al., 2006).

The origin of American foulbrood is unknown. According to Matheson (1993), the disease is widespread, except in vast regions in Africa. The absence or the low level of American foulbrood in the large territory of sub-Saharan Africa is probably due to the primitive beekeeping, the hygienic behavior and physiological sensitivity of African bees, and their propensity to abandon the nest in irritations, provoked by pests or sudden changes in the environment (Fries and Raina, 2003). However, in countries with temperate-continental climate, where the disease is widely distributed, the situation is different (Genersch, 2010; Pernal and Melathopoulos, 2006). In Bulgaria, American foulbrood is a notifiable disease, and beekeepers receive compensations for each destroyed bee family after microbiological confirmation of the causative agent. For this reason, data from the regional structures is entered on the national register of the Bulgarian Food Safety Agency (BFSA), on an annual basis.

Recently, a variety of *P. larvae* genotypes were discovered, exclusively using the molecular techniques

PFGE, rep - PCR and ERIC-PCR. Until now, a total of six rep-PCR genotypes have been found in Germany - AB, Ab, ab, αB, aβ, AБ (Genersch and Otten, 2003; Peters et al., 2006). In a study on 214 *P. larvae* strains from Austria with rep - PCR, Loncaric et al. (2009) differentiated another two genotypes - aB and αb, which had not previously been detected with this technique. Knowing the genotypes is an important epidemiological tool for American foulbrood risk assessment due to the established correlation between genotype, virulence of the strains and the development of clinical symptoms (Loncaric et al., 2009).

The lack of published data on the prevalence of American foulbrood in Bulgaria was the motivation to perform the present study. What is more, a genetic *P. larvae* map of Bulgaria has been produced.

Materials and Methods

Data on the number of American foulbrood outbreaks and the destroyed bee colonies from the register of the Bulgarian Food Safety Agency, Animal Health Directorate, for the last 25 years (1989 - 2013) were collected and analyzed. The incidence of American foulbrood in South-and North Bulgaria was studied in the period 1998 - 2013 considering the number of outbreaks (1998 - 2008) and destroyed bee families (2009 - 2013) for the two regions of the country.

A total of one hundred and seven *P. larvae* isolates originating from fifteen apiaries were isolated and genotyped using rep - PCR with BOXA1R and MBOREP1 primers as previously described (Rusenova et al., 2013). Brood samples were collected between 2009 - 2014. Three of the apiaries were located in North Bulgaria (37 *P. larvae* isolates), the rest of apiaries were situated in the south regions (70 *P. larvae* isolates).

The significance of American foulbrood prevalence between South and North Bulgaria, was evaluated by the means of t-test (GraphPad Soft Inc., USA 2003).

Results

Data on the number of American foulbrood outbreaks and the destroyed bee colonies in the period 1989 - 2013 are visualised in Figure 1. Most outbreaks, respectively destroyed colonies were registered at the beginning of the period when 350 outbreaks were recorded in 1989 (4800 families) and 271 (1910 families) in 1992. The following years 1993 - 2008 were characterized by a significant reduction in the number of outbreaks and destroyed colonies, with a marked wavy pattern and a peak in 2006, when 175 outbreaks were recorded and 763 bee colonies were destroyed. In the last five years of the reporting period, there was a tendency towards reduction in the number of destroyed families (n = 32 - 76), with an increase to 102 in 2013.

The prevalence of American foulbrood in South and North Bulgaria is shown in Figure 2. It is evident that over the period, the number of outbreaks and destroyed families was considerably higher in the southern part of Bulgaria (P<0.01). Only in 2000 and 2012, was their number slightly higher in North Bulgaria.

The location of outbreaks caused by the established *P. larvae* genotypes ab and AB is presented in Figure 3. Overall, 25 AB *P. larvae* isolates were detected in 5 apiaries, as the only genotype in 4 apiaries and two AB isolates were found in combination with ab in one apiary. The majority of AB isolates (n = 23) originated from outbreaks in the southern Bulgaria. The ab genotype was found in 11 apiaries.

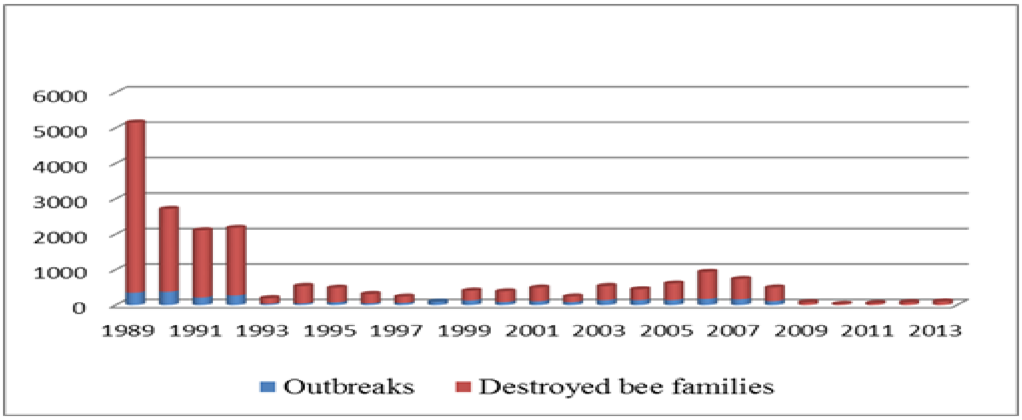

Figure 1. Prevalence of American foulbrood in Bulgaria in 1989 - 2013.

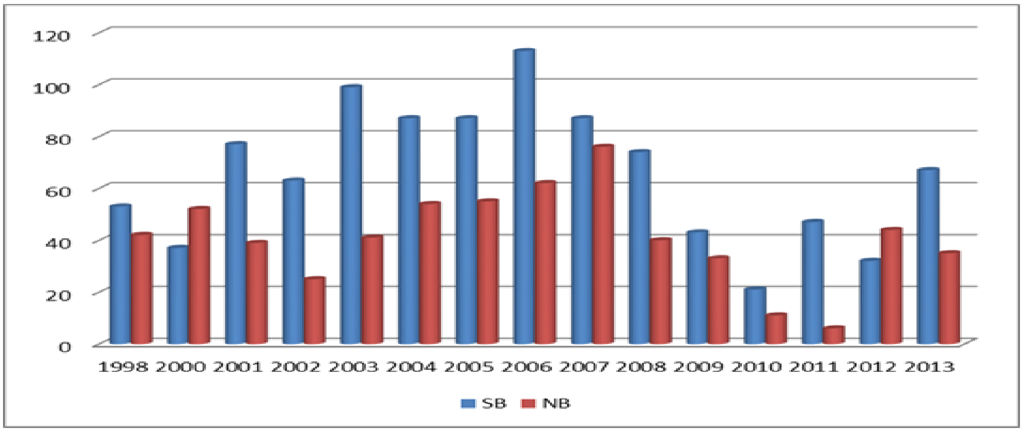

Figure 2. Prevalence of American foulbrood in South (SB) and North Bulgaria (NB) in 1998 - 2013.

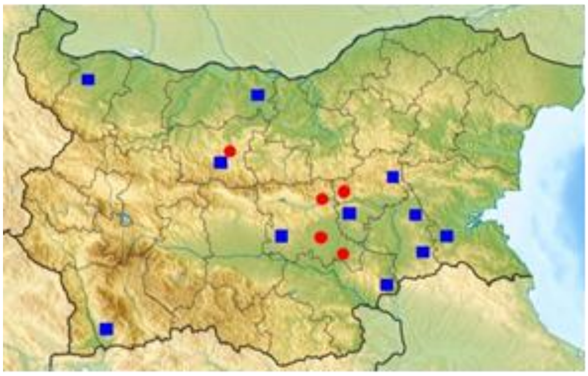

Figure 3. Location of AFB outbreaks in Bulgaria with determined *Paenibacillus larvae* genotypes.

Legend: *P. larvae* ab genotype ■
P. larvae AB genotype ●

Discussion

The results from this survey showed a strongly irregular pattern of American foulbrood spread over the reported period. The highest number of affected bee families, respectively American foulbrood foci, was detected in 1989 - 1992 as a result of the actively working schedule for disease control at that time. According to Parvanov et al. (2004) the transition to market-oriented economy in these years forced beekeepers to move the apiaries in settlements for security reasons and the increased density was a prerequisite for spread of various pathologies, including American foulbrood. Another reason is the still active system for American foulbrood control during that period, which implied mandatory prophylactic examinations of bee families in the country and reporting of the real epidemiological situation every spring and autumn. From 1993 to 2008, the BFSA archive records demonstrated a substantial reduction of affected apiaries and bee families. However, in our opinion, this reflects the diminished veterinary control at the time and the collapsed system of control rather than a lower prevalence of the disease. There is no information on the BFSA register about the number of affected bee families in 1998, and the last five years of the period 2009 - 2013, as indicated in Figure 1. The official register of the BFSA should keep records not only for the destroyed bee families affected by American foulbrood, but above all, about disease foci by regions, which would facilitate the monitoring and the control of the disease.

Signs of American foulbrood are most commonly manifested in late summer and in the autumn (Parvanov et al., 2006). The strongest bee families in the apiary are usually the first to be affected. The reason being that strong families easily find the weakened ones, rob the honey and bring the infection in their nests (Lindström et al., 2008). Apart from the natural pathways, apiculture practices such as feeding contaminated honey, the transmission of combs with brood, and the use of infected beekeeping tools are also important for the transfer of spores among bee families. Furthermore, keeping beeswax in storehouses accessible for bees, and trading with bee families and queens are also significant for the transmission of spores (Mutinelli, 2011). The disease is also present in neighbouring countries. In Turkey, American foulbrood is established in 9 - 13% of all regions, but in 2010 - 2011 the occurrence was 17% (Özkirim et al., 2011). A study on 31 apiaries in western Romania has demonstrated American foulbrood only in 2% of brood samples (Balint et al., 2011).

The genotyping of *P. larvae* is of special significance for epidemiological labelling and tracing of the infection source (Genersch and Otten, 2003; Loncaric et al., 2009). This is the first study showing the genetic map of *P. larvae* in Bulgaria. As seen from Figure 3, most isolates with genotype AB originated from apiaries in South Bulgaria. In biological experiments, Genersch et al. (2005) and Schild et al. (2014) confirmed a stronger virulence of the AB genotype vs both ab and Ab genotypes. Bee larvae infected with *P. larvae* AB perish more rapidly and before capping of cells, nursing bees clean the cells and thus, reduce the spores in the nest. Therefore, the spread of this genotype in the nest occurs more slowly and the clinical diagnosis is complicated by the scarce specific findings in the comb. As a result, the unrecognized AB genotype could spread and affect other bee families (Genersch et al., 2005). The predominance of the AB genotype in apiaries from southern regions in the country could explain the more severe course of the disease in South Bulgaria (Bonovska et al., 2014). Genersch and Otten (2003) and Peters et al. (2006) have also established a specific geographical distribution pattern of *P. larvae* genotypes in Germany. Unfortunately, our study did not include samples from all regions of the country, despite our efforts and contacts with regional subdivisions of the Bulgarian Beekeepers Union – the National Branch association and Regional Veterinarians. Most probably, beekeepers prefer to destroy the affected bee families rather than inform the competent authorities, declaring officially the outbreak and seeking compensation after laboratory confirmation of the disease. The lack of samples from all regions of the country did not allow us to make final conclusions about the prevalence of genotypes of American foulbrood causative agent in Bulgaria.

In conclusion, the analysis of the data demonstrated that although the number of affected bee families over the last years was small (about 700,000 bee families are available in the country), the detection of foci of infection at a national level requires a perfect monitoring, management including regular mandatory prophylactic examinations of bee families with respect to early diagnostics, identification of genotypes of the pathogen and American foulbrood control.

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