Investigation of *Paenibacillus larvae* subspecies larva of honey bee (*Apis mellifera*) colonies in East Azerbaijan Province of Iran

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Abstract:

BACKGROUND: The American foulbrood disease (AFB), caused by *Paenibacillus larvae* subsp. larvae, is one of the main plagues affecting honey bee (*Apis mellifera* L.) colonies, and it has a high negative impact on beekeepers worldwide. **OBJECTIVES:** The aim of this research was to describe the evidence of *Paenibacillus larvae larvae* contamination of honey bee colonies, in the East Azerbaijan Province. **METHODS:** In this study, samples from honey bee colonies of North West Iran were studied for their respective ability to detect the presence of bacterial contamination, using microbiological methods. For this purpose, 10 ml of the suspension from live bees, larva, honey, pollen and wax were incubated in modified MYPGP agar (Muller-Hinton broth-yeast extract-glucose-sodium pyruvate and PO4HK2) plates. Bacterial colonies were identified by colony, bacteria morphology and biochemical tests for *Paenibacillus larvae larvae*. **RESULTS:** The results of this study indicated a low level of contamination with *Paenibacillus larvae larvae* in the East Azerbaijan Province. **CONCLUSIONS:** In conclusion, the distribution of *P.l.larvae* spores in all samples of the East Azerbaijan Province, showed a clear pattern and may provide useful data for the control and prevention of American foulbrood.

Introduction

American foulbrood (AFB) in honey bees (*Apis melifera*) is caused by the spore forming bacterium, *Paenibacillus larvae larvae* (Nordstrm et al., 2002). AFB is one of the few bee diseases capable of killing a colony, and is difficult to prevent and control because the spores can remain viable for long periods of time (Alippi and Reynaldi, 2006). In many countries, AFB is a notifiable disease and most authorities consider burning infected or diseased colonies, the only workable control measure against AFB (Yue et al., 2008). In countries where disease incidence is high, the use of antibiotics appears as an alternative to the burning of infected bee hives (Alippi and Reynaldi, 2006, Alippi et al., 2002). AFB is a serious problem in apiculture and causes considerable economic loss to bee keepers all over the world (Genersch, 2010). Spreading of the disease within an apiary and between apiaries or even countries is facilitated by beekeeping practice like exchanging materials between colonies, managing numerous hives in a confined area and global trading of bees and honey (Ashiralleva and Genersch, 2006).

American foulbrood can be diagnosed in the field by visual inspection; however, field – di-

agnosis must be microbiologically confirmed. Microscopic identification can be complemented by the modified hang-drop technique, the Holst milk test or fluorescent – antibody techniques developed for detection of *P. larvae* antigens (Abd Al-Fattah et al., 2010). Besides, the most frequently applied identification method is the isolation of P.larvae followed by biochemical characterization tests (Steinkraus and Morse, 1996). For biochemical diagnosis of suspected P.larvae colonies, catalase and nitrate reduction tests are recommended (Abd Al-Fattah et al., 2010).

Recently, field observations on Iranian apiaries showed foulbrood symptoms tentatively related to AFB. Very few reports have recorded limited AFB infections in Iran (Yusefkhani and Lotfi, 2010). This research investigated the occurrence of AFB in honeybee colonies in East Azerbaijan Province. Diagnosis of the disease symptoms was preliminarily carried out by relying on visual inspection and field tests verified by isolation and phenotypic identification of the pathogen.

Materials and Methods

Sample collection: Inspectors from the North West of Iran (Azerbaijan) were asked to collect samples of honey, wax, adult bee, larva and pollen. For this purpose, 10 ml of each suspension (wax, adult bee, larva and pollen) was incubated in each of the six modified MYPGP agar plates. One hundred colonies, without any detectable symptoms but from apiaries with diseased colonies, were sampled during the years 2010-2011 for the detection of AFB in the apiary.

Sample analysis (Samples of adult bees): Each adult bee sample, consisting of 50 worker bees, was placed in a polythene bag (10× 15 cm) with an inner nylon mesh (1.2 × 1.2 mm) and 20 ml of sterile NaCl (9 mg/ml) was added. The bag was sealed using a heat sealer and bees in the sealed bag were carefully crushed by hand. The fluid was collected into a 50 ml centrifuge tube and centrifuged at 27000 g (Ependorff) for 10 min. The resulting pellets were resuspended in sterile NaCl to a total volume of 2 ml and incubated at 85°C for 10 min in a water bath (Nordstrm et al., 2002).

Samples of honey: Five grams of each honey sample was diluted with 5 ml of sterile NaCl (9 mg/ml). The honey samples were incubated at 88°C for 10 min (Nordstrm et al., 2002, Pernal and Melathopoulos, 2006).

Samples of wax: One gram of wax was added to the test tube with airtight seal. Larger pieces of wax were cut by sterile instruments into very small pieces. The dry material prepared in this way was stirred thoroughly and diluted with 8.5 ml of sterile distilled water. The resulting suspension was then supplemented with 0.5 ml of Tween 80. Careful homogenization resulted in the development of a homogenous, grayish brown pulpy material which hardened on cooling. Afterwards, the tubes were removed from the water bath and allowed to cool down to room temperature at which they were stored for 2-4 h, until a sufficient amount of liquid was separated at the bottom of the tubes. Then, 2-5 ml of this liquid was withdrawn with a disposable balloon pipette and mixed with the same volume of distilled water in another sterile sealable tube. Again, the resulting mixture was shaken thoroughly in the longitudinal direction for at least 5 min and put into a hot water bath. After 10 min, the tubes with the stand were removed from the bath, allowed to cool down to room temperature and shaken again (Bzdil, 2007).

Samples of pollen: In total, 0.75 g of pollen samples were suspended in 2.5 ml of sterile 0.9% NaCl and homogenized by hand in a glass tissue grinder (Gilliam et al., 1989).

Samples of larvae: Larvae were taken out of their cells, washed twice in distilled water and touch- dried lightly on filter paper. Eppendorf vials were filled with 8 larvae each. After the addition of 60 µl of sterile distilled water,

Table 1. Biochemical characteristics of American foulbrood bacteria isolated from East Azerbaijan province. VP: Voges-Proskauer.

Identification	Gram's test	Spores forming	Hydrolysis of gelatin	Production of indole	Reduction of nitrate	VP reaction	Oxidase	Catalase
14	+	+	+	-	+	-	+	-
10	+	+	+	-	+	-	+	-
8	+	+	+	-	+	-	-	-
4	+	+	+	-	+	-	+	-
12	+	+	+	-	+	-	-	-

the contents were homogenized by ultrasonic treatment (Crailsheim and Riessberger-Galle, 2001).

Enumerating samples: Ten microliters (10 µl) of the suspension was incubated in each of the six modified MYPGP agar plates. The plates were incubated at 36°C in 5% CO2 for 7 days, after which the number of bacterial colonies were counted. Bacterial colonies were identified by colony and bacteria morphology and a negative catalase test for *Paenibacillus larvae larvae*. Production of Indole, nitrate reductase (Abd Al-Fattah et al., 2010), gelatinase, oxidase and acetyl methyl carbenol (Vogas- Proskauer test) were also examined. More than 100 colonies in each plate were counted. Contamination with bacteria other than *Paenibacillus larvae larvae* were noted for each plate and sample (Nordstrm et al., 2002).

Results

During the years 2010-2011, 97 out of 100 beekeepers had no colony of P. larvae.

The analysis of honey, larva and wax samples shows that just one sample is positive for P. larvae. The analysis of bee samples showed that two samples were infected with *P. larvae* and there was no infection with *P. larvae* in pollen samples.

Samples collected from the same area of the hive showed that honey, wax, larva and bee could be representative of the colony at the time of sampling, when no symptoms were found. Consequently, measures can be taken to prevent the establishment and further dissemination of the disease (de Graaf et al., 2006).

A total of five Gram-positive spore-forming bacteria were isolated from the samples collected from all the infected locations. Isolates were purified and their cultural, morphological as well as biochemical traits were examined (Table 1).

Discussion

Sampling of honey bees has been previously documented to be a very sensitive technique, for the detection of AFB, especially for individual colonies (Nordstrm et al., 2002; Ritter, 2003; Lindstrom, 2008). The honey samples have a limitation when applied at the apiary level, these limitations are not greater than those of other methods used for monitoring the transmission or prevalence of *Paenibacillus larvae larvae* spores. On the contrary, because the spore load of adult bees reflects the actual status of the colonies at the time of sampling, rather than the status at the time of nectar collection, it gives a more accurate picture of the conditions in the apiary and greater possibilities to correlate clinical disease to the spore load. Wax is also a good sample. As spores of *Paenibacillus larvae larvae* remain infectious even for decades, the danger of infection persists also from abandoned apiaries free of bees. It can only be excluded by examining wax samples (Ritter, 2003).

Different *P. larvae* identification rates have been reported from the suspected colonies. Yusefkhani and Lotfi (2010) reported that 5.8% of honey bees in the Northwest of Iran were detected as positive for *P. larvae* infection. This percentage is similar to the one found in

this work. Of the 36 honey samples tested for AFB in Moharrami's research (2011), a high level of infection was present in 13.9%.

Findings regarding the occurrence of *P. larvae* infections in foreign apiaries are highly varied. Among the total samples found in Kilic's research (2008), a high level of infection was found in 100 samples (7%). In Uruguay (Antunez et al., 2004), the first clinical case of AFB was found in 2000 samples. In 2001 and 2002, during tests of 101 honey samples from 19 provinces, *P. larvae* was found in 52 samples (51.5%). In Africa, Fries and Raina (2003) tested 64 samples of imported honey and 35 samples of African honey. They detected *P. larvae* in six samples of imported honey. In Poland, following tests of 6510 samples of honey, the research showed the presence of *P. larvae* in 35.6% of the honeys (Pohorecka et al., 2012).

The results suggest that, at least under Azerbaijan conditions, contaminated adult bee may act as reservoir of *P. larvae* spores. If bees cannot defecate outside the hive and rely solely on the honey stores for food, there is a buildup of fecal matter mixed with *P. larvae* spores in the rectum of adult bees and such spores remain viable. If bees defecate inside the hive, spores are released into the hive environment. This probably increases the risk of larval infection due to a more spore-contaminated environment (Lindstrom et al., 2008). The distribution of *P.l.larvae* spores in all samples of the East Azerbaijan Province showed a clear pattern and may provide useful data for the strategy of control and prevention of American foulbrood.

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بررسی میزان آلودگی زنبورستان‌های استان آذربایجان شرقی با باکتری پنی باسیلوس لاروlلاروl

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چکیده

زمینه مطالعه: بیماری لوک آمریکایی که توسط *Paenibacillus larvae larvae* ایجاد می‌شود، یکی از مهمترین عوامل ایجاد پلاک می‌باشـد که کندوهای زنبور عسـل را درگیر کرده و باعث تحمیل خسارات اقتصادی به پرورش دهندگان زنبور عسل در سراسر جهان می‌شود. **هدف:** هدف از این مطالعه، مقایسه نمونه‌های مختلف در مراکز پرورش زنبور عسل از نظربررسی میزان آلودگی به باکتری *Paenibacillus larvae larva* در زنبورسـتان‌های اسـتان آذربایجان شرقی می‌باشد. **روش کار:** در طی این مطالعه، نمونه‌های زنبور، لارو، عسل، گرده و موم به دست آمده از شمال غرب ایران از نظر آلودگی به باکتری مذکور با روش کشت میکروبی مورد مقایسه قرار گرفتند. بدین منظور ۱۰ µL از سوسپانسیون حاصل از زنبور، لارو، عسل، گرده و یا موم بر روی محیط پلیت MYPGP کشت شدند. پرگنه‌های باکتریایی با استفاده از شکل پرگنه و شکل باکتری و نیز منفی بودن تست‌های بیوشیمیایی مورد شناسایی قرار گرفتند. **نتایج:** نتایج این مطالعه نشان داد که اگر هدف جستجوی میزان آلودگی به باکتری *Paenibacillus larvae larvae* در کندوهای زنبور عسل استان آذربایجان شرقی در حد بسیار پایینی می‌باشد. **نتیجه‌گیری‌نهایی:** براساس نتایج بدست آمده در این مطالعه، به نظر می‌رسد کشت از نمونه‌های مختلف مراکز پرورش زنبور عسل معیار مناسبی جهت بررسی آلودگی به بیماری لوک آمریکایی تهدید بزرگی برای زنبورستان‌های استان آذربایجان شرقی به حساب نمی‌آید.

واژه‌های کلیدی: لوک آمریکایی، نمونه زنبور، نمونه عسل، نمونه لارو

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