

## **Preliminary Investigation on American Foulbrood Disease: 1. Recording the Infection in the Apiaries at Fayoum Governorate**

**Ayman A. Owayss**

Plant Prot. Dept., Fac. Agric., Fayoum Univ., Egypt. E-mail: ayman\_owayss@hotmail.com

### **Abstract**

American foulbrood (AFB) is one of the most destructive diseases of the honey bee. This infectious disease causes a great economic loss in beekeeping industry worldwide. This severe brood infection is caused by the spore forming bacterium, *Paenibacillus larvae* subspecies *larvae* White (formerly: *Bacillus larvae* subsp. *larvae*). AFB was not detected in Egypt till about two years, however the disease was suspected in certain Egyptian governorates (Giza, Gharbia and Beni-suef), but no legitimated information or authorized reports were published. Recent inspection of suspect brood samples obtained from apiaries situated in Tameia and Ibshawai districts (Fayoum governorate) showed typical symptoms of AFB. Subsequent diagnostic chemical, microbiological and biological assays ascertained the incidence of AFB in the tested samples. This may be the 1<sup>st</sup> time authenticated AFB infection in Fayoum gov. Migratory beekeeping, importing of bee products and unauthorized bee queens from infected countries are possible vectors of the disease.

**Key words:** American foulbrood disease – Honey bees – Fayoum Governorate – Egypt.

### **Introduction**

American foulbrood (AFB) disease, caused by the microaerophilic, gram-positive, spore forming bacterium *Paenibacillus larvae* (formerly: *Bacillus larvae*) (Heyndrickx *et al.*, 1996) is the most virulent brood disease infecting honeybees (*Apis mellifera*) (Bailey, 1985 and Shimanuki *et al.*, 1992). The disease causes severe damage to the beekeeping industry worldwide by decimating the infected bee colonies. Only the spore stage of *P. larvae* can initiate infection. Larvae, less than 3 days old, are susceptible to the disease. Spores are ingested in contaminated food and then germinate in the midgut. The resulting vegetative rods further multiply in the gut lumen, then penetrate the gut

epithelium, and enter the hemocoel, where they circulate with the hemolymph. Endospores are formed and released from the vegetative rods. As the disease progresses, the white larvae change color and die from septicemia. The remains eventually dry up to become "scales", which may be adhered to the wax cells. The spores can survive on the combs and on contaminated equipments and tools almost indefinitely. By resulting the contaminated equipment or tools, beekeepers can easily spread the disease (**Williams *et al.*, 1998**). The longest survival test conducted showed that the spores are capable of germinating after 35 years in scales. It is, however, expected that the spores can survive much longer (**Hansen and Brødsgaard, 1999**).

Recently, in Egypt, there were some indications about the occurrence of AFB in some regions without formal authentication, but with some exceptions *e.g.* **Zakaria (2007)**, in Giza, Egypt, mentioned that during summer season of 2006, 10 honey bee colonies (hybrid Carniolan) of 75 ones were infected with AFB. The present work aimed to ascertain the presence of AFB in Fayoum gov.

## **Material and Methods**

### **1-Samples of suspected brood**

Two apiaries (150 & 100 colonies) of *Apis mellifera carnica*, hybrid honey bees situated in Ibshawai district, and three other apiaries (80, 90 & 100 colonies) situated in Tameia district, Fayoum gov., Egypt, were inspected for AFB infection. The suspect infection ranged between 30-50% in these apiaries. Brood combs from infected colonies were carefully wrapped and obtained to the Fac. Agric., Fayoum Univ. These combs were kept at – 5°C till required diagnosis tests.

### **2-Diagnosis of the disease**

#### **2-1- Symptoms:**

To diagnose symptoms of AFB, the steps given by **Shimanuki and Knox (1991)** were followed concerning appearance of brood, age of dead brood, color, consistency and scale characters.

## **2-2- The milk test:**

According to **Holst (1949)**, this test based on the fact that a high level of proteolytic enzymes is produced by sporulating bacteria. The test was conducted by suspending a suspect scale or smear of a diseased larva in a test tube containing 3-4 ml of 1% powdered skim milk in water. The tube was then incubated at 37°C. If the causative bacteria is present, the suspension should clear in 10-20 min. A blank test tube should be carried out.

## **2-3-Culturing of microorganism:**

Ten smears of infected honey bee larvae were homogenized in 1ml sterilized water and pipetted to 1.5 ml Eppendorf tube. To culture the causative agent of AFB (*P. l. subsp. larave*), this suspension was spread over petri-dishes containing brain heart infusion (Difco) fortified with 0.1 mg thiamine hydrochloride per liter of sterilized medium (BHIT) and adjusted to pH 6.6 with HCL. Then, the plates were incubated at 34°C for 72 hours (**Shimanuki and Knox, 1991**).

To obtain good vegetative growth and sporulation, the medium (1g yeast extract, 1g glucose, 1.35g K<sub>2</sub>PO<sub>4</sub>, 1g soluble starch and 2g agar in 100 ml) recommended by **Bailey and Lee (1962)** was also compared. After pH was adjusted to 6.6, the medium was autoclaved at 10 lb/sq.in. at 116 °C for 20 min. After inoculation of the plates with larval suspension, they were incubated 34°C for 72 hours. Characters of bacterial colonies including form, elevation, color, margin, optical of color, diameter and texture were recorded.

## **3- Isolation of the causative pathogen**

The bacterial colonies grown on culturing media were recultured, separately in new plates and incubated at the same conditions. Pure bacterial colonies were inoculated in a liquid medium (agar-free) and prepared for about 5% inoculum. Slants for different isolates were also prepared for subsequent identification

#### **4- Pathogenicity test:**

To ascertain the infection with the isolated causative agent, pathogenicity test was carried out. According to **Hornitzky (1998)**, with some modifications, 5% bacterial inoculum was sprayed on bee brood combs (*A. m. carnica*) larvae (2-3 day-old) reared in small nuclei. Later, developed symptoms were recorded. Inoculum-free suspension was also applied as control.

#### **5- Identification of the causative pathogen**

The bacterial isolate responsible for AFB symptoms which occurred in pathogenicity test was identified with the microbiological assays. The steps to ascertain the infection and the pathogen given by **Hornitzky (1998)** was followed.

### **Results and Discussion**

#### **1-Diagnosis of the disease**

Inspection of sealed worker brood showed the discoloration sunken and punctured cappings (plate, 1). Larvae under their cappings were upright in cells. The consistency of dead brood was soft and sticky (plate, 2). The color of dead larvae was dull white (plate, 3), coffee brown to dark (plate, 4), larval content was watery when burst (plate, 5) and with pronounced glue odor to rotten fish odor. Scale of dead brood were flat on lower sides of cells, adhere tightly to cell wall. The milk test (**Holst, 1949**) was also positive. These findings are the most typical symptoms of AFB (**Shimanuki, 1990; Shimanuki and Knox, 1991 and Hansen and Brødsgaard, 1999**).

#### **2- Identification of the causative pathogen**

Two specific media were used to isolate the responsible bacterium for AFB in the tested samples. Bacterial colonies (plates, 7 - 10) were into similar colonies (plates 11 & 12). Identification steps including colony characters, and microscopical examination (100x oil immerse) were carried out.. The AFB causative bacteria (*P. l. larvae*) is a slender rod with slightly rounded ends and a tendency to grow in chains. The rod varies greatly in length (2.5-5.0  $\mu\text{m}$ ) and is about 0.5  $\mu\text{m}$  wide. The spore is oval and approximately twice as long as wide

(0.6x1.3µm). When stained with carbol fuchsin, the spore walls appear reddish purple and quite clear in center (**Bailey and Lee, 1962; Alppi, 1991; Shimanuki and Knox, 1991, and Peters *et al.*, 2006**).

According to the above results, and as a subject for future work, subsequent research must be done to:

Determine the level of infection and epidemiology of the disease.

Detect and identify the other associated microorganisms (using PCR).

Control strategy must be achieved on short and long term to protect beekeeping industry from this dangerous disease.

Also, extension programs must be carried out for beekeepers to be aware about all information related to AFB.

**Acknowledgement:** I'm indebted to **Dr. Khaled A. El-Banna**, Lecturer of Agric. Microbiology at the same Faculty, for his generous help and guidance through identification steps..

### **References**

- Alppi, A. M. (1991). A comparison of laboratory techniques for the detection of significant bacteria of the honey bee, *Apis mellifera* in Argentina. *J. Apic. Res.*, 30 (2): 75-80.
- Bailey, L. (1985). "Honey Bee Pathology", pp. 26-30. Academic Press, San Francisco, CA, USA.
- Bailey, L. and Lee, D. C. (1962). *Bacillus larvae*: its cultivation in vitro and its growth in vivo. *J. Gen. Microbiol.*, 29, 711-717.
- Hansen, H. and Brødsgaard, C. J. (1999). American foulbrood: a review of its biology, diagnosis and control. *Bee World*, 80 (1): 5-23.
- Heyndrickx, M.; Vandemeulebroecke, K.; Hoste, B.; Janssen, P.; Kersters, K.; De Vos, P.; Logan, N. A.; Ali, N. and Berkeley, R. C. (1996). Reclassification of *Painebacillus* (formerly *Bacillus*) *pulvifaciens* (Nakamura, 1984) Ash *et al.*, 1994, a later subjective synonym of *Paenibacillus* (formerly *Bacillus*)

- larvae* (White, 1906) Ash *et al.*, 1994, as a subspecies of *P. larvae*, with emended descriptions of *P. larvae* as *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens*. Int. J. System. Bacteriol. 46: 270-279.
- Holst, E. C. (1949). A single field test for American foulbrood. American Bee Journal, 36:14, 34.
- Hornitzky, M. (1998). The pathogenicity of *Paenibacillus larvae* subsp. *larvae* spores and vegetative cells to honey bee (*Apis mellifera*) colonies and their susceptibility to royal jelly. J. Apic. Res., 37 (4): 267-271.
- Peters, M.; Kilwinski, J.; Beringhoff, A.; Reckling, D. and Genersch, E. (2006). American foulbrood of the honey bee: Occurrence and distribution of different genotypes of *Paenibacillus larvae* in the administrative district of Arnsberg (North Rhine-Westphalia). J. Vet. Med. B 53: 100-104.
- Shimanuki, H. (1990). Bacteria. In Honey Bee Pests, Predators and Diseases. 2<sup>nd</sup> ed. 27-47 pp. Morse, R. A. and Nowogrodzki, R. (eds.). Cornell University Press, London, UK.
- Shimanuki, H.; Knox, D. A. (1991). Diagnosis of Honey Bee Diseases. US Department of Agriculture, Agric. Handbook No. AH-690, 53p.
- Shimanuki, H.; Knox, D. A.; Furgala, B.; Canon, D. M. and Williams, J. L. (1992). Diseases and pests of honey bees. In " The Hive and the Honey Bee" (J. M. Graham, Ed.), pp. 1083-1096. Dadant, Hamilton, IL.
- Williams, J. R.; Peng, C. Y. S.; Chuang, R. Y.; Doi, R. H. and Mussen, E. C. (1998). The inhibitory effect of Azadirachtin on *Bacillus subtilis*, *Escherichia coli*, and *Paenibacillus larvae*, the

causative agent of American foulbrood in the honeybee, *Apis mellifera*. J. Inverteb. Pathol., 72: 252-257.

Zakaria, M. E. (2007). The cellular immunity response in the haemolymph of honey bee workers infected by American foulbrood disease (AFB). J. Appl. Sci. Res., 3 (1): 56-63.



Plate. (1). Punctured sealed brood of tested comb.



Plate (2). Sticky consistency (thread) of dead larva.



Plate (3). Honey bee larvae infected with AFB.



Plate (4). Prepupae infected with AFB.





Plate (5). Destructive content of infected larvae.

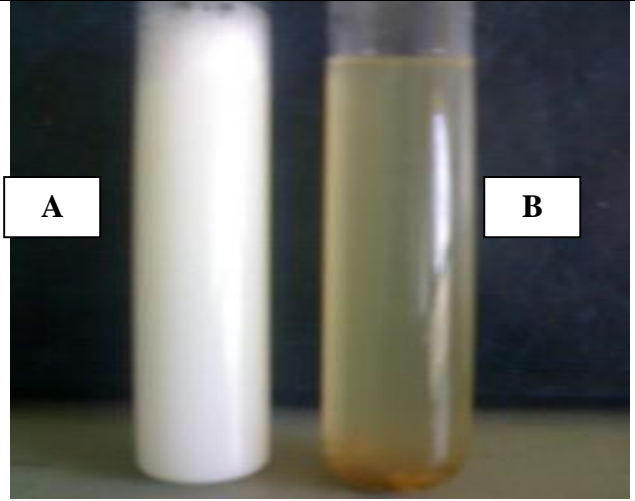


Plate (6). Milk test: A= blank and B = positive.

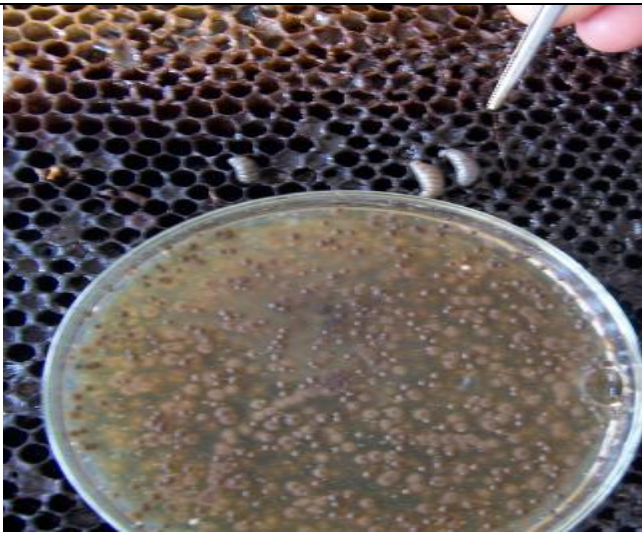


Plate (7). Bacterial growth compared to comb.

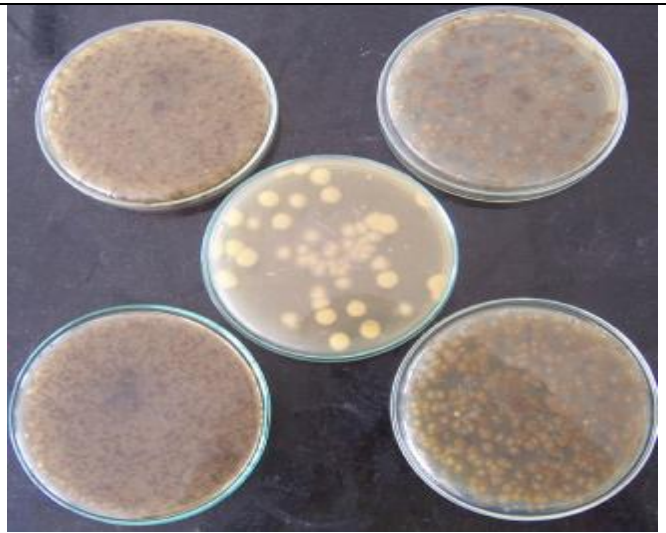


Plate (8). Different bacterial isolates from cadavers.



Plate (9). Pure culture of a bacterial isolate.

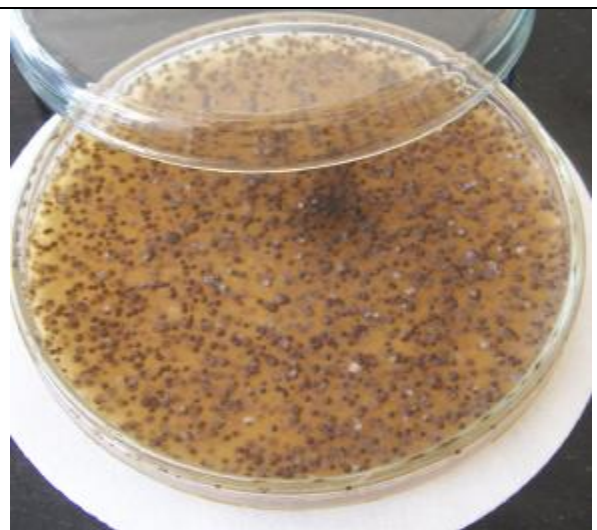


Plate (10). Dominant growth of a bacterial isolate.





Plate (11). Growth on Bailey and Lee medium.

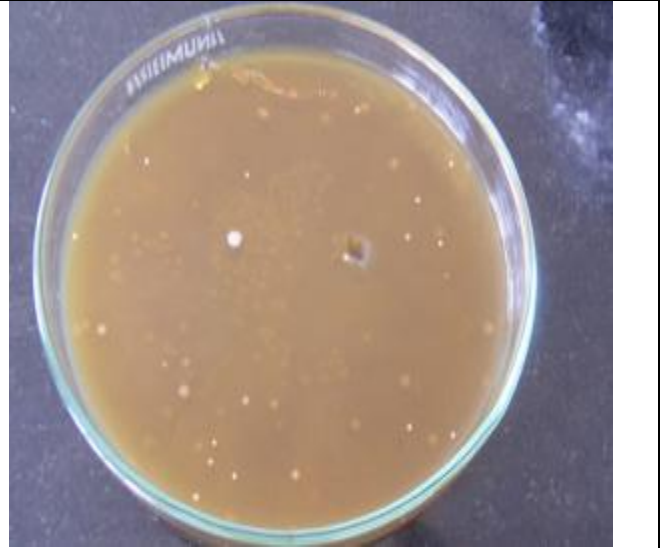


Plate (12). Growth on BHIT medium.

## دراسة مبدئية لمرض تعفن الحضنة الأمريكي: 1. تسجيل الإصابة في مناطق محافظة الفيوم

أيمن أحمد عويس

قسم وقاية النبات - كلية الزراعة - جامعة الفيوم - مصر.

E-mail: ayman\_owayss@hotmail.com

### الملخص

يعد مرض تعفن الحضنة الأمريكي واحدا من أخطر الأمراض التي تصيب نحل العسل ويسبب خسائر فادحة على مستوى العالم. يدمر هذا المرض حضنة النحل وتسببه البكتيريا المتجرثمثة *Paenibacillus larvae*. ولم يسجل هذا المرض في مصر من قبل، ولكن قبل حوالي عامين ظهرت بعض الدلائل التي تشير إلى وجوده في بعض محافظات مصر (الجيزة، الغربية، بنى سويف)، إلا أنه لم تنشر تقارير رسمية أو موقعة تؤكد أو تنفي الإصابة بهذا المرض. وبفحص عينات حضنة، وجدت في بعض مناطق مركزى طامية، و أبشواى بمحافظة الفيوم، وجدت الأعراض المميزة لهذا المرض. كما أكدت الدراسات الكيماوية، والميكروبيولوجية، والبيولوجية التي أجريت على هذه العينات وجود الإصابة وتأكيدا، ربما لأول مرة في محافظة الفيوم.

واستنادا إلى هذه النتائج، وكموضوع دراسة مستقبلية يجب:

1- أن يستمر البحث لمعرفة مدى وبائية المرض وانتشاره على مستوى الجمهورية لاتخاذ كل ما يلزم لحماية مهنة تربية النحل المهتدة بهذا الؤافء الجءءء.

2- عمل برامج توعية للنحالءن وعرض نتائج هذه الأبحاث عليهم لءكونوا على دراية بكل ما ىءءلق بهذا المرض.

الكلمات الدالة: مرض تعفن الحضنة الأمريكي - نحل العسل - الفيوم - مصر.