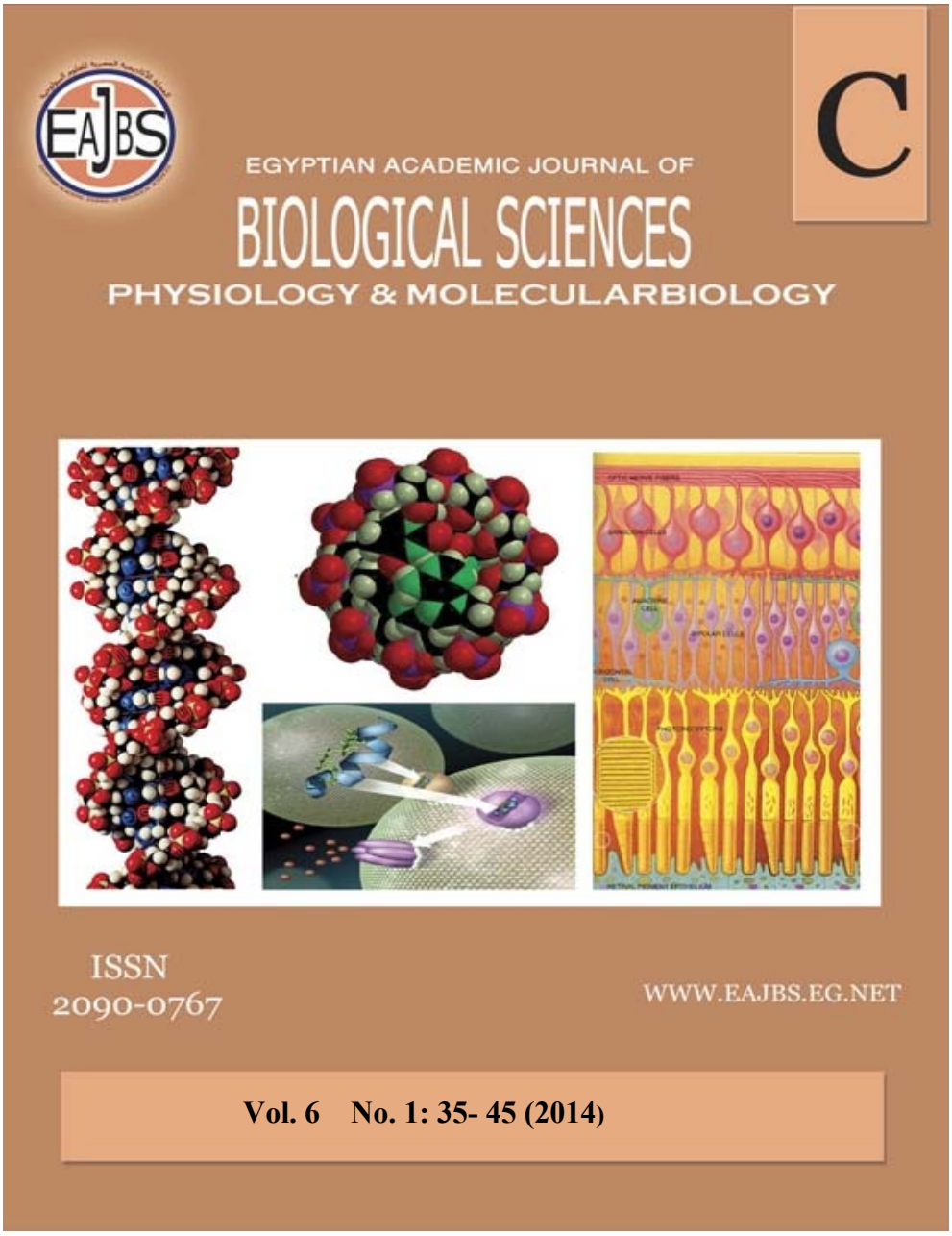


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# Molecular characterization of *Paenibacillus larvae larvae*, for early diagnosis of american foulbrood of honeybees in Egypt

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**ABSTRACT**

A reliable procedure for early detection of *Paenibacillus larvae* subsp. *larvae* (*P. l. larvae*), the causal agent of American Foulbrood disease (AFB) of honeybees (*Apis mellifera* L.) based on the polymerase chain reaction (PCR) and subspecies – specific KAT primers. A PCR amplicon of the expected size 550 bp only found in *P. l. larvae* strains was used for positive AFB. This PCR assay provides a specific detection for *P. l. larvae* from week 1 post infection even if there is no clinical symptoms appeared in a colony.

The technique can be directly used to detect presence or absence of *P. l. larvae* spores in honeybee samples and contaminated honeys.

## INTRODUCTION

The honeybees *Apis mellifera* is an extremely beneficial insect due to its role in pollination and for its products (honey, wax, probolis, pollens and venom). Hence, apiculture has a great economic impact; health status of honeybees has become an important concern in many countries.

Bees are constantly under threat due to combined damage from bacteria, parasites, viruses, pesticides, insecticides, and artificial bee food (Cox-Foster *et al.*, 2007, Stokstad, 2007, Aliouane *et al.*, 2008, Higes *et al.*, 2008).

The most common bacterial disease which is lethal at the honeybees' larval stages, is the American foulbrood (AFB) disease. It is caused by an endospore-forming, Gram-positive rod-shaped bacterium, *P. l. larvae*, that infects young larvae through ingestion of contaminated food (Shimanuki, 1997).

Geographical origin of AFB is unknown, but it is found almost world-wide (Matheson, 1993, 1996). AFB is the most virulent brood disease known in honeybees (*Apis mellifera* L.). It is one of the few bee diseases capable of killing a colony and possess unique problems for prevention and control because the bacterial spores can remain viable for long periods of time (35 years or more) and survive adverse conditions (Matheson and Reid, 1992).

Symptoms of infection are easily recognizable in freshly dead larvae (Alippi *et al*. 1999). Methods to be applied for diagnosis of AFB depend on clinical characteristics. In an advanced state of illness, AFB affected brood are characterized by the glutinous consistency of the larval remains, which can be drawn out as threads with a thin device. However, the diagnosis must be confirmed by laboratory tests. Microscopic identification of stained bacteria (Michael, 1957), the Holst milk test (Holst, 1946), or fluorescent-antibody techniques developed for the detection of *P. larvae* antigens (Toshkov *et al.* 1970, Zhavnenko 1971, Otte 1973). Besides microscopy, the most frequently applied identification method is the isolation of *P. larvae* using culture media followed by characterization by biochemical tests. For biochemical diagnosis of suspected *P. larvae* colonies, the catalase (Haynes, 1972) and nitrate reduction (Loch head, 1937) tests are widely used. When clinical signs are absent or information on the appearance of the brood is missing (for instance, the examination of honey or wax) the identification of the pathogenic agent demands a more profound identification of suspicious colonies (de Graaf *et al*, 2006).

Molecular techniques have also been developed for the identification of *P. larvae* (Alippi and Aguilar, 1998a, b). Govan *et al*. (1999) and Dobbelaere *et al*. (2001) have described PCR assays for the detection of *P. larvae*-specific DNA in bacterial colonies grown on semi-selective medium. This technique was suggested for use in the rapid confirmation of the presence of *P. larvae* strains isolated from honey samples. Alippi *et al*. (2002) described a method, based on PCR and restriction fragment analysis, which allows the differentiation of *P. l. Larvae* strains from all other bacterial species.

It will be very useful to develop a molecular technique for the early diagnosis of AFB disease in Egypt before its outbreaks. This will make a new profile that can be used in the diagnosis of different honeybee diseases in Egypt, in order to enhance the apiculture of different honeybee traits for higher productivity and easier rearing. This technique is used not only for diagnosis but also for identification of the bacterial pathogen *P. l. larvae* subspecies and its strains. So this technique could be used in detection of *P. l. larvae* strains present in Egypt.

The present work aims to early diagnosis of the American foulbrood disease before clinical symptoms appearance using molecular techniques.

## MATERIALS AND METHODS

The experiment was set up in apiary yard of the Apiculture Research Department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Egypt, during the summer 2011. A total of 5 honeybee Colonies were used, with 2 colonies left untreated and used as control. The control colonies were all in other sites from infected colonies. The other three colonies were given a honeycomb from a heavily AFB infected colony.

Adult bees were sampled once a week for the first 4 weeks and then once a month until appearance of the disease signs. The bees were chosen from combs in the brood nest. Adult bee samples, consisted of >15 adult bees from each colony, were collected into a plastic bag. The bags were sealed and samples were stored at (-20°C) until used.

Honey samples were taken from honeycombs weekly for 4 weeks post-infection and then monthly until appearance of the disease signs, when sampling was terminated. Samples were taken by scooping with the edge of a falcon tube along the surface of different parts of the comb. In the colonies where contaminated honeycombs had been inserted, the samples were taken from other honeycombs.

**Isolation and Cultivation of the bacterial pathogen *P. l. larvae*:**

Ropy larval remains of died honeybee larvae (collected from naturally infected colony) were suspended in 10 ml sterile

distilled water and kept at room temperature for 10 min, then the suspension is heat-shocked at 80 °C for 10-15 min (effective time to kill non-spore-forming bacteria).

For the isolation of *P. l. larvae*, after vortex mixing, 1ml of the bacterial stock suspension is directly inoculated onto J-agar (5.0 gm tryptone, 15.0 gm. yeast extract, 3.0 gm. $K_2HPO_4$, 2.0 gm. glucose, 20.0 gm. agar, 1000 ml of distilled water "Adjust pH to 7.3-7.5" Shimanuki and Knox 1988) by using the pour-plate technique. The plates were incubated in an inverted position at 35 ± 2 °C for 48 hours.

A number of individual colonies were randomly selected from the inoculated plates, depending on the individual colonies characteristics. The selected colonies then inoculated onto J-agar plates by using the streak-plate technique, following the same incubation conditions (Piccini and Zunino, 2001). Initial identification assessing colony characteristics, microscopic characterization and standard biochemical tests (Alippi, 1992).

**DNA preparation and manipulation:**

For bacterial DNA preparation from cultured colony, a part of the colony was suspended in 50 µl bidistilled water and subsequently incubated at 90 °C for 15 min. Probes were centrifuged at 5000 g for 10 min (Kilwinski *et al*, 2004). The supernatant containing the DNA was transferred to a new tube and directly used for PCR analysis.

For bacterial DNA preparation from honeybee workers five adult bees were crushed in a fine mesh inside a plastic bag with 5 ml sterile water. The liquid was poured into centrifugal tube and centrifuged at 27,000 G for 10 min. the supernatant was discarded and the pellet was resuspended in 50 µl of sterile distilled water. DNA was prepared as previously mentioned for cultured colony.

For honey samples, 10g of honey was diluted in 10 ml of sterile distilled water and the solutions were incubated at 95°C for 6 min. Thereafter, 10 ml of each solution was centrifuged at 4,000 g for 30 min (Bakonyi *et al*, 2003) and the pellet was used for DNA extraction as previously mentioned.

**Primer pair and PCR conditions:**

The pair of primers KAT1 and KAT2 (Alippi *et al*. 2002) of sequences: 5'-ACAAACACTGGACCCGATCTAC-3' and 5'-CCGCCTTCTTCATA TCTCCC-3', respectively were used. The reaction mixture (25 µl) contained 2.5 µl buffer 10x (Promega) 20 µM of KAT1 and KAT2 each forward and reverse primers; 3mM MgCl (Promega ); 2mM of each dNTP (Promega); 1U Taq DNA polymerase and 1µl of DNA obtained as described above.

The optimal temperature cycling conditions were adjusted according to Alippi *et al*. (2002). The cycling program consisted of a 94 °C (5min) step, 30 cycles of 94 °C (30s), 63.5 °C for annealing (1min), 72 °C (1.30 min), and a final step of 72 °C (5min). Amplifications were performed in a thermal cycler Techne (TC 312). The PCR products were separated electrophoretically in a 1% agarose gel at 100 V for 30 min, stained with ethidium bromide and photographed on an u. v. transilluminator using an Olympus D-760 16 megapixels digital camera.

## RESULTS

Clinical symptoms were observed in the weekly inspections. One of the treated colonies developed clinical disease symptoms on the third week post infection and for the other two colonies; clinical symptoms appeared on the fourth week.

DNA extracted and amplified from honeybee workers and honey samples showed 550 bp amplicon for positive AFB. Contrarily, no signal was noticed for negative AFB disease.

**PCR results for week1:**

First week post infection, honeybees and honey samples collected from treated apiarian were examined by using DNA KAT-PCR.

PCR results of the honeybee samples showed an identical positive pattern among the three treated colonies. Whereas, the honey samples had the positive pattern in only two colonies (Fig.1).

In order to assess the usefulness of the PCR assay with primers KAT1 and KAT2, we run the PCR-reaction on DNA extracted from pure bacterial colony, cultured from larval remains which showed clear clinical signs of AFB, as positive control and also from samples of honeybee and honey from untreated honeybee colonies, as negative control (Figs. 1, 2, 3 and 4).

**PCR results for week 2, 3, and 4:**

For the second, third and fourth week, all honeybee and honey samples from treated colonies resulted positive for the 550 bp amplicon, giving positive results (Figs. 2, 3, 4).

Table (1), summarizes PCR results of all colonies, infected and control, for the first four weeks post-infection. All honey and honeybee samples have shown positive results except only one honey sample, collected from honeybee colony number 3 on the first week post infection, showed negative result (*P. l. larvae* not detected).

Table 1: KAT-Pcr detection of *Panaebacillus larvae larvae* from honey and honeybee samples collected from infected and control colonies for the first 4 weeks post-infection.

| **Colony** / ***P. l. l.* Pcr diagnosis from** | | **Infected** | | | **Control** | |
|---|---|---|---|---|---|---|
| | | **Colony 1** | **Colony 2** | **Colony 3** | ***Colony 4*** | **Colony 5** |
| **Honey** | **Week 1** | + | + | - | - | - |
| | **Week 2** | + | + | + | - | - |
| | **Week 3** | + | + | + | - | - |
| | **Week 4** | + | + | + | - | - |
| **Honey bee workers** | **Week 1** | + | + | + | - | - |
| | **Week 2** | + | + | + | - | - |
| | **Week 3** | + | + | + | - | - |
| | **Week 4** | + | + | + | - | - |

*+ = presence of ***Panaebacillus larvae larvae*** specific PCR band.
*- = absence of ***Panaebacillus larvae larvae*** specific PCR band.

## DISCUSSION

The most common method for detection of AFB is visual inspection of the brood combs for clinical symptoms (Shimanuki, 1997).The clinical symptoms of AFB are typical, with the brown, viscous larval remains forming a ropy thread when drawn out with a matchstick. It has an unpleasant odor that sometimes can be noticeable. The decaying larvae desiccate into hard scales, consisting of millions of bacterial spores.

In an infected colony, spores from *P. larvae* can be isolated from honey, wax, pollen, and hive walls (Gochnauer, 1981). It has been reported that the *P. larvae* spores can remain infective for at least 35 years (Haseman, 1961). The disease spreads when spores are transported on drifting bees, hive parts, clothing, and contaminated pollen or honey (Delaplane, 1991). The examination of honey for spores may therefore be of value in tracing disease outbreaks, and there have been a number of studies using honey for this purpose (Hansen, 1984, Alippi, 1995). The detection of these inapparent or latent infections would identify sources of pathogens which may cause fully developed disease in these hives or spread of infection to other hives.

It is well-known from field observations that some colonies show no clinical symptoms despite a high spore concentration contaminating the honey,

while others exhibit clinically diseased brood although the spore concentration detectable in the honey is low (Hansen and Brødsgaard 1999). So far, these differences have been explained by differences in host tolerance and hygienic behavior of honeybees (Woodrow 1942, Woodrow and Holst 1942 and Hansen and Brødsgaard 1999).

Reliable detection methods are also of great importance for studies of pathogen transmission within and between colonies. Of the methods available today, adult bee sampling has been shown to reflect the current disease status of the colony most correctly (Nordström *et al*., 2002). However, the method needs further evaluation at different organisational levels to determine its usefulness and limitations both for practical screening purposes as well as for epidemiology and transmission studies.

Although honey has been widely used to Monitor for AFB infections (Hansen 1984), our results indicated that culturing of colony level composite samples of adult bees is more sensitive than culturing of honey samples from the same colony this is in agreement with the results obtained by Nordström *et al.* (2002) on infected honeybee colonies. Hornitzky and Karlovskis (1989) introduced the method of culturing adult honeybees for AFB and demonstrated that spores could also be detected from colonies without clinical symptoms. These false positives represent colonies that are infected by the pathogen but where clinical symptoms are not manifested. From an epidemiological perspective, such subclinical infections should not be neglected because they may be responsible for considerable horizontal disease transmission within beekeeping operations, also where clinical disease symptoms have not appeared, as beekeepers move material between colonies. Adult bees from the brood chamber has been reported to contain more spores per bee compared with samples from the supers (Goodwin *et al*. 1996), but the differences are small and may not be of practical importance when sampling for diseased colonies (Lindström and Fries 2005). This is in agreement with our results, we preferred to take bee samples from brood chambers and this gives 100 % AFB detection all over the four weeks post infection.

Whereas our honey samples produced false-negative results (no PCR-bacterial detection in spite of its presence in the honeybee colony), samples of adult bees have not produced false negatives when sampling of adult bees, and field inspection for clinical symptoms have been done at the same time. This was also mentioned by (Lindström and Fries 2005) in their field studies and culturing of bacterial samples.

The detection of contaminated honey plays an important role in the efficient control of American foulbrood. The small numbers of spores and the presence of other *Paenibacillus* and *Bacillus* species complicate the identification of *P. larvae* in honey samples. That's why honey may produce false-negative results in AFB detection. Also large-scale screening of honey samples for *P. larvae* by classical isolation and identification methods is rather time-consuming, laborious, and expensive.

PCR is a quick and reliable method that is widely used in microbiological diagnostics for the detection of specific nucleic acid sequences in biological materials. In this study, we tested KAT-PCR system for the detection of *P. larvae*-specific DNA in honeybees and honey and test the PCR assay's sensitivity for the diagnosis of the AFB disease from week one post infection until the appearance of symptoms.

Govan *et al.* (1999) described a PCR detection method for rapid identification of *P. larvae*. Their system was developed for the identification of *P. larvae* cultivated from honey samples in semi-selective medium (and not for direct detection in honey). These primers detected *P. larvae* nucleic acid at a level of 10 CFU and failed to detect *P. larvae* DNA in honey samples.

Alippi *et al*. (2004) designed the pair of oligonucleotides KAT1 and KAT2, which was assayed as primers in the PCR reaction we used. They extracted and purified DNA

template from *P. l. larvae* as well as from the most common bacteria and fungi found to be present in apiarian samples. The limit of detection of the PCR system when *P. l. larvae* DNA was used was 15 ng of crude DNA. All the *P. l. larvae* strains produced a PCR amplicon of the expected size of 550 bp whereas the other bacteria and fungi samples yielded no PCR-product. This result confirms those described above in which a specific profile in fingerprinting and DNA hybridization as well, were found associated to *P. l. larvae* strains.

Primers KAT1 and KAT2 also specifically detected *P. l. larvae* in larvae samples as well as its spores in adult honeybee and honey samples. The limit of detection in honey for this PCR method is 1 CFU per isolation plate that correlates with 17 viable spores per gram of honey which is equivalent to 283 total spores per g of honey, which takes into consideration that only 6% of the total spores are able to germinate in MYPGP medium (Dingman and Stahly 1983).

However, the issue of false negatives is a problem that depends on the desired detection level and how large a sample one is prepared to collect and process. The spores of *P. larvae* are not randomly distributed among adult bees. This distribution is important to know the sample sizes when colonies are monitored for AFB, using PCR assays of the DNA of the causative agent from adult bees. The presented results strongly suggest that samples of adult bees from individual colonies are highly effective in detecting clinically diseased colonies.

In this study we investigated quite intensively the applicability of PCR for the detection of *P. larvae*, the causative agent of American foulbrood, and found certain PCR assays to be appropriate for quick screening of honey samples for the presence of *P. larvae*.

Our study provides a method for distinguishing *P. larvae* subsp. *larvae* in Egypt. Direct detection of spores instead of the isolation of vegetative forms may save time used for germination and growth. Our method determines quickly and unambiguously the presence of *P. larvae*. The complete procedure takes less than 4 h.

We believe this method can be applied for the reliable and rapid diagnosis of AFB, which may facilitate the screening of honey and apiarian materials, and aid the production of honey in a *P. l. larvae*-free environment.

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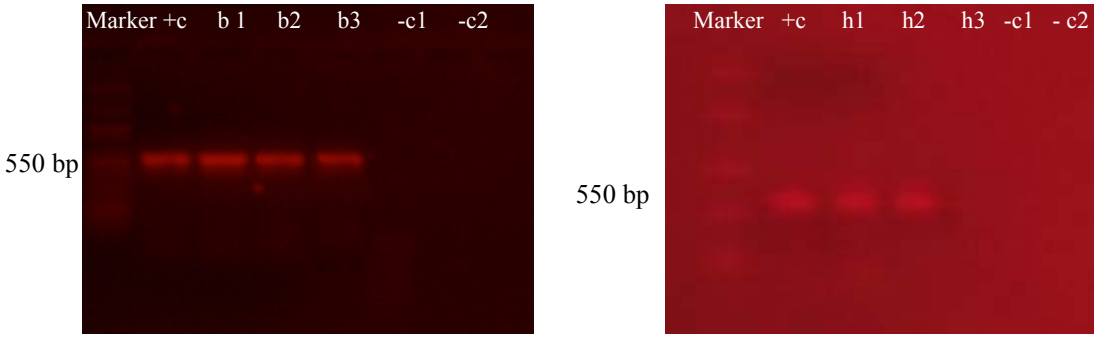
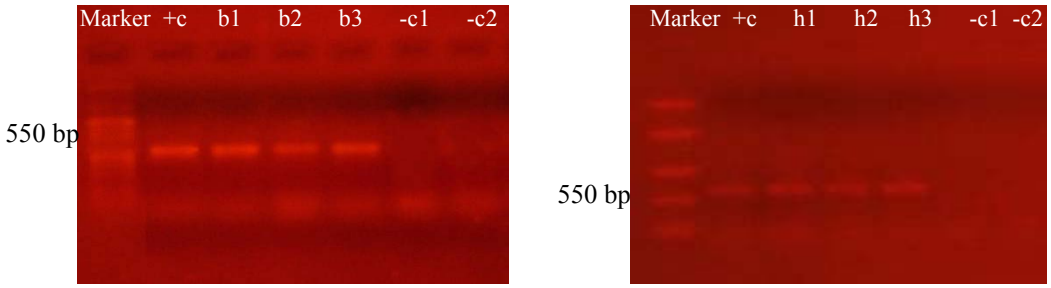


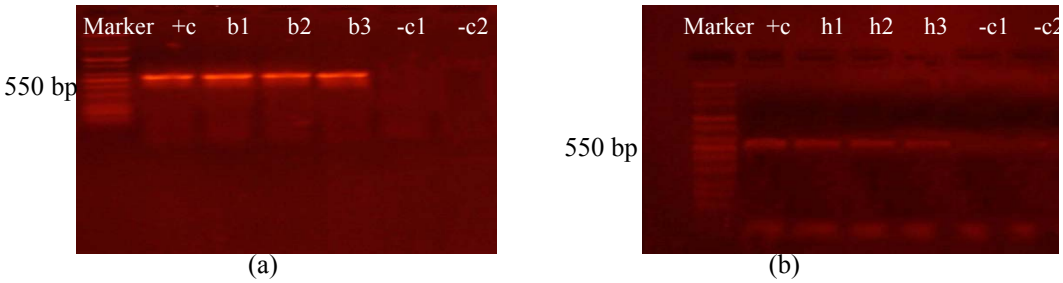

(a) (b)

**Fig. 1** First week post infection ***P. larvae*** subspecies ***larvae***-specific PCR products of 550 bp obtained by using primers KAT1 and KAT2 (a) honeybee samples: +c= positive control, b1= honeybee samples from treated colony 1, b2= honeybee samples from treated colony 2, b3= honeybee samples from treated colony 3, -c1= honeybee samples from untreated colony negative control 1 and –c2= honeybee samples from untreated colony negative control 2 and (b) honey samples: +c= positive control, h1= honey samples from treated colony 1, h2= honey samples from treated colony 2, h3= honey samples from treated colony 3, -c1= honey samples from untreated colony negative control 1 and –c2= honey samples from untreated colony negative control 2.



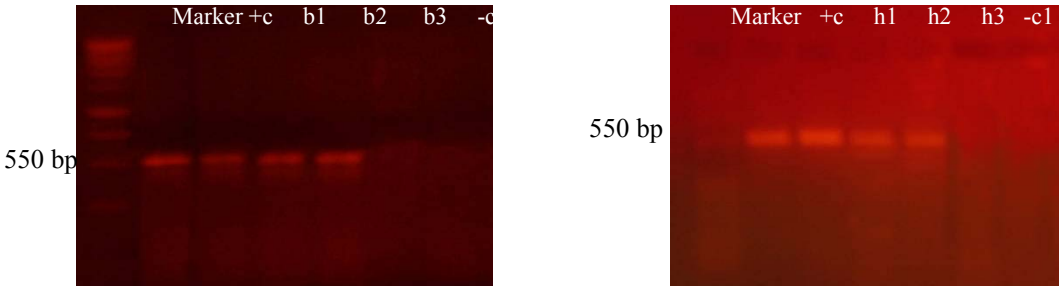

(a) (b)

**Fig. 2** Second week post infection ***P. larvae*** subspecies ***larvae***-specific PCR products of 550 bp obtained by using primers KAT1 and KAT2 (a) honeybee samples: +c= positive control, b1= honeybee samples from treated colony 1, b2= honeybee samples from treated colony 2, b3= honeybee samples from treated colony 3, -c1= honeybee samples from untreated colony negative control 1 and –c2= honeybee samples from untreated colony negative control 2 and (b) honey samples: +c= positive control, h1= honey samples from treated colony 1, h2= honey samples from treated colony 2, h3= honey samples from treated colony 3, -c1= honey samples from untreated colony negative control 1 and –c2= honey samples from untreated colony negative control 2.




(a) (b)

**Fig. 3** Third week post infection ***P. larvae*** subspecies ***larvae***-specific PCR products of 550 bp obtained by using primers KAT1 and KAT2 (a) honeybee samples: +c= positive control, b1= honeybee samples from treated colony 1, b2= honeybee samples from treated colony 2, b3= honeybee samples from treated colony 3, -c1= honeybee samples from untreated colony negative control 1 and –c2= honeybee samples from untreated colony negative control 2 and (b) honey samples: +c= positive control, h1= honey samples from treated colony 1, h2= honey samples from treated colony 2, h3= honey samples from treated colony 3, -c1= honey samples from untreated colony negative control 1 and –c2= honey samples from untreated colony negative control 2.




(a) (b)

**Fig. 4** Forth week post infection ***P. larvae*** subspecies ***larvae***-specific PCR products of 550 bp obtained by using primers KAT1 and KAT2 (a) honeybee samples: +c= positive control, b1= honeybee samples from treated colony 1, b2= honeybee samples from treated colony 2, b3= honeybee samples from treated colony 3, -c1= honeybee samples from untreated colony negative control 1 and –c2= honeybee samples from untreated colony negative control 2 and (b) honey samples: +c= positive control, h1= honey samples from treated colony 1, h2= honey samples from treated colony 2, h3= honey samples from treated colony 3, -c1= honey samples from untreated colony negative control 1 and –c2= honey samples from untreated colony negative control 2.

## ARABIC SUMMARY

# إستخدام التعريف الجزيئي *للبانيباسيلاس لارڤي لارڤي*، في التشخيص المبكر لمرض تعفن الحضنة الأمريكي فى نحل العسل فى مصر

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الهدف من هذه الدراسة هو استحداث طرق جديدة للتشخيص المبكر لمرض تعفن الحضنة الامريكي فى نحل العسل (*أبيس ميللفرا* لينيس) فى مصر. تم ذلك عن طريق تجميع عينات عشوائية من شغالات نحل العسل (الطور البالغ) وعسل النحل من الخلايا غير المصابة والخلايا المعدية بالمرض إسبوعيا في الأربع أسابيع الأولي التى تلت العدوى حتى ظهور الأعراض الممر ضة لليرقات. طبقت فى هذه الدراسة طريقة تكبير تفاعل البلمرة المتسلسلة باستخدام البادئKAT لمنطقة محددة في جين (16s rRNA) والموجود في الحمض النووى (دنا) والمعروف إستخدامها فى تعريف البكتيريا المسببة للمرض(*بانيباسيلاس لارڤي لارڤي*) . وقد أوضحت الدراسة أن طريقة الـ (KAT-PCR) تعطي نتائج سريعة ومؤكدة لوجود العامل المسبب للمرض في شغالات نحل العسل وعسل النحل وذلك قبل ظهور الأعراض الممرضة للحضنة من الأسبوع الأول للعدوي بالبكتيريا المسببة للمرض.