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Diagnosis of *Paenibacillus larvae* Honeybees Disease in Jordan Using PCR Techniques

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Abstract
American foulbrood disease (AFB) is considered one of the most virulent bacterial diseases of honeybee (*Apis mellifera Linnaeus*, 1758*)* it has a vital negative impact on the beekeeping industry worldwide. This disease is caused by a spore forming bacterium *Paenibacillus larvae* that affects honeybee larvae. This work includes the subsequent diagnosis including microbiological and PCR procedure for detection of *P. larvae*. Fifty-six honey and larval samples were collected from 56 inspected honeybee colonies located in 53 apiaries representing beekeeping all over Jordan. The samples were examined to assess the presence of *Paenibacillus larvae* in honey samples by using both microbiological methods and PCR techniques. *Paenibacillus larvae* was isolated from 41.6% samples (honey and larval) using bacteriological methods and 38.5% using PCR techniques. PCR were effectively used to amplify a specific region of the genome of *P. larvae* from pure culture isolates, honey and larvae samples; amplified regions include: Enterobacterial Repetitive Intergenic Consensus (ERIC) and 35KDa Metalloprotease. The positive PCR samples were collected in Amman, Irbid, Al-Ghor, Al-Zarqa and Al-Balqa.

Keywords : American foulbrood, *Apis mellifera*, ERIC , Honey,

Introduction
Honeybees are important not only for the honey they produce but also as vital pollinators of agricultural and horticultural crops. It has been estimated that one-third of the human diet can be traced directly or indirectly to honeybee pollination. The decline in pollinators is a global threat to biodiversity. The main purpose of apiculture in Jordan is honey production because of the high price of the local honey. Although honey production is of economic significance, the contribution of bees in pollination of agricultural plants has not been acknowledged yet in Jordan [1]. Most diseases that affect honeybees should be considered serious and a few are lethal not only to the individual bees but to the whole colony [2,3].
Throughout the world, one of the most severe honeybee diseases is American Foul Brood [4]. This disease causes significant economic losses to the beekeeping industry worldwide and its prevention and control are problematic because bacterial spores remain viable for long periods [5].
American foulbrood disease (AFB) is considered one of the most virulent bacterial diseases of honeybee (*Apis mellifera*) and is known to have a negative impact on the beekeeping industry worldwide. This disease is caused by a spore forming bacterium *Peanibacillus larvae* that affects honeybee larvae. AFB was not officially detected in Jordan till about three years ago [7, 8], but no legitimated information or authorized reports were published.

Molecular techniques (PCR) have been developed for the identification of *P. larvae* [8, 10]. Govan et al [11] developed the first PCR assay of identification of *P. larvae*, he described the detection of *P. larvae*-specific DNA in bacterial colonies grown on semi-selective medium. Followed by another PCR assay by Dobbelaere et al [10] who described a similar test in a higher specificity, he found better and simplified protocol for immediate analysis of DNA extracts of the remains of AFB diseased larvae.
The objectives of this work were the diagnosis and identification of *Peanibacillus larvae* in different regions of Jordan using PCR techniques.

1. Materials and methods:

1.1. Samples collection.
Fifty-six honey samples were collected from different apiaries throughout Jordan during the spring and the summer, as shown in table (1). They were taken from 56 inspected honeybees colonies according to a preliminarily field diagnosis by beekeepers, located in 53 apiaries. From these samples, sixty of them were honey (brood nest and bulk) samples originating from six governorates (Irbid, Ajloun, Amman, Al-Balqa', Az-Zarqa, and Al-Shobak). The five samples were larvae samples originating from Irbid and Amman

Table 1: The apiaries site, number of apiaries and number of collected samples

Apiaries location	No. of Apiaries	No. of Brood-nest Honey Samples	No. of Bulk Honey Samples
Amman	13	4	6
Al-Shobak	3	5	0
Irbid	25	25	2
Al-Ghor	6	9	0
Alzarqa (Hashemite University)	1	4	0
Al-Balqa	5	5	0
Total	53	52	8

1. 2 Bacteriological method diagnosis of AFB Disease
MYPGP is the culture medium used for the diagnosis and isolation of the bacterium. MYPGP agar: 1.5% (wt/vol) yeast extract, 1% (wt/vol) Mueller-Hinton broth from Oxiod (England), 0.3% (wt/vol) K_2HPO_4, 1% (wt/vol) sodium pyruvate, 0.2% (wt/vol) glucose. (Autoclaved separately) and 2% (wt/vol) agar [12, 13].

1.2.1 Isolation of P .larvae from AFB infected Honey samples
Samples were heated to 45°C in a water bath for 5 min to permit easy handling and to decrease viscosity and allow more distribution of spores. 25 ml of honey was then placed in 50 ml tubes, and diluted (1:1) with 25 ml of sterile phosphate buffered saline. After vigorous mixing, the suspension was centrifuged 20 min at 27.000 rpm by ultracentrifuge to harvest the spores. Then, the pellet was re-suspended by SDW to reach 1.5 ml volume and heat shocked at 80 °C for 15 min.
Samples for brood nest honey were carried out by direct inoculation thought by taking 5 ml of the honey samples diluted (1:1 v/v) with sterile distilled water , the solutions incubated for 15 min at 80°C (heat shocked), then the suspension was ready to inoculate on MYPGP [14].

1.2.2. Isolation of P. larvae from AFB infected larvae.
Larvae with clinical symptoms of AFB were removed by using a toothpick and then mixed well with 1 ml of SDW (2 larval remains per tube) .100 µl of the suspension was diluted in 900 SDW, vortex mixed, then the suspension centrifuged 2000 rpm for 5 min.[3]. 200µl from each sample were inoculated over the surface of MYPGP, and then incubated at (34-37) °C for 2-4 days (CO_2 incubator, USA).

1.3 DNA extraction
1.3.1. Enrichment of P. larvae
Bacterial colonies were collected with a sterile inoculation loop and were dispersed in five ml of MYPGP broth (2X concentrated: 1.5% (wt/vol) yeast extract, 1% (wt/vol) Mueller- Hinton broth, 0.3% (wt/vol) K2HPO4, 1% (wt/vol) sodium pyruvate, 0.2% (wt/vol) glucose (autoclaved separately) with the addition of Nalidixic acid (

final concentration 12mg/ml). The suspension was incubated at 37°C for 24 hrs with continuous shaking at 170 rpm in the dark [14].

1.3.2. DNA Extraction from MYPGP broth

Bacterial colonies that were enriched with MYPGP broth were harvested in a microfuge tube by centrifugation at 14,000 rpm for three min. The collected pellets were resuspended in 300µl of CTAB buffer solution (2% (wt/vol) CTAB, 1.4M NaCl, 1% (wt/vol) PVP, 0.02M EDTA and 0.1M Tris-HCL. (pH adjusted to 8.0), and incubated in water bath at 65°C for 30 min with continuous shaking, then the bacterial lysate was incubated with 30 µl of 1mg/ml RNase enzyme at 37°C for 30 min. After that equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) was added, mixed and microcentrifuged at 14.000 rpm for 5 min. The supernatant was then transferred to a new tube. Afterward, an equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added, mixed, and micro- centrifuged at 14.000 rpm for 5 min. The supernatant was transferred again to a new tube, 0.6 volume isopropanol were added and mixed gently and then incubated at - 60°C for 30 min or overnight at -20°C until DNA precipitated. Then, the tube was centrifuged at 14.000 rpm for 15 min and the supernatant was discarded and the precipitated DNA was washed with 1 ml of 70 % ethanol, centrifuged at 14.000 for 5 min., supernatant were discarded and DNA was dried at room temperature after inverting the tubes on a filter paper to allow all the fluid to dry away. Finally, the pellet was resuspended in 50-µl TE buffer (10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0), and stored at -20°C until used .

1.3.3. Purity of DNA

Quality of DNA was tested by running 10µl of DNA solution mixed with 2µl of loading dye (Promega®, USA) in 0.7 % agarose gel at 80 volt for 30 min, and then photographed (Alphalamager®, USA).

1.4. PCR Amplification

The amplification technique can be directly used to detect presence or absence of *P. larvae* spores in honeybee brood samples and contaminated honeys. Two primers were used to amplify two specific regions of *P. larvae* . (Table 2)

Table 2: Primer pairs used to amplify a portion of ERIC region and 35DKa

Primers	Sequences	Product size	References
KAT1 KAT2	5'-ACAAACACTGGACCCGATCTAC-3 5'-CCGCCTTCTTCATATCTCCC-3'	550-bp	Allipi et al (2004) [8]
PII-MP F3 PII-MP B1	5'-CGGGCAGCAAATCGTATTCAG-3' 5'-CCATAAAGTGTTGGGTCCTCTAAGG-3'	273-bp	Neuendorf et al (2004) [14]

1.4.1. Amplification of ERIC region of P. larvae

Allipi research team [8] designed the pair of oligonucleotides KAT 1 and KAT 2, with 550 bp amplification region of the *P. larvae* ERIC region using the extracted DNA. PCR analyses were carried out in a final volume of 25 µl, consisting of 1X Taq DNA polymerase buffer (Torbay Road, Markham L3R 1G, Canada), 2.5 µM of each primerv (Alpha DNA, CO., Montreal, Canada), 3 mM MgCl2, 0.32 mM deoxy nucleotide triphosphate (dNTPs) (Bio Basic, Canada), 2 µl DNA, and 1U Taq DNA polymerase. The PCR was carried out on a thermal cycler (Pelter, DNA Engine, USA), according to the following protocol: one cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 63.5°C for 1 min, and 72°C for 1 min and 30 s; then final extension step at 72°C for 5 min. PCR products were kept at -20°C for further use.

1.4.2. Amplification of 35-Dka metalloprotease for P. larvae

The primer pair Pll-MPF3/ Pll-MP B1 [13] was used to amplify a portion (273 bp) of the *P. larvae* Metalloprotease precursor gene (accession number AF111421). (Table 2). The 25µl mixture of the PCR reaction consist of 1X Taq DNA polymerase buffer (Bio Basic, Canada), 1.5 mM MgCl2, and
10 µM of each primer (Alpha DNA, CO., Montreal, Canada), 0.25 mM dNTPs (Bio Basic, Canada), 1 µl DNA, and 2U Taq DNA polymerase (Bio basic, Canada). The metalloprotease precursor gene was amplified according

to the following protocol: After the initial activation step (15 min, 95 ºC), the reaction conditions for the touchdown PCR were as follows; the denaturation steps were performed at 94°C for 30 s, elongation at 72 ºC for 30 s, and annealing for 1 min. For annealing, temperatures of 66, 62 and 58 ºC were used and 5 cycles were run at each temperature. At the final annealing temperature of 56 ºC, 30 cycles were run followed by a final elongation step at 72 ºC for 8 min. PCR products were kept at -20ºC for further use.

1.5. Detection of Amplified PCR Products

Agarose gel electrophoresis was performed as described by Dobbelaere et al [9]. Aliquots 10 µl of each PCR products were electrophoresed on a 1-1.5 % agarose gel using 0.5X TBE buffer (pH 8.3: 54g Tris base, 27.5g Boric acid and 20ml of 0.5M EDTA). The PCR products were visualized with an UV transilluminator, photographed with the gel documentation system (Bio-RAD, gel electrophoresis, France) after staining the gel with ethidium bromide (0.5 µg/ml). The DNA molecular weight marker, 100 bp DNA ladder (Promega, CO., Madison, WI, USA) was used to determine the size of the amplified Fragments.

2. Results and discussion

2.1. Amplification of the ERIC-region of P. larvae by PCR

The template DNA was extracted from bacterial colonies of *P. larvae* using boiling and CTAB method. 25 out of 32 (78%) suspected *P. larvae* colonies produced a defined band of 550 bp. The rest of other samples cannot be detected when PCR performed on DNA isolates.

2.2. Amplification of the 35KDa Metalloprotease Precursor of P.larvae by PCR

Positive *P. larvae* colonies by ERIC-based PCR test that gave the expected size of amplicon 550 bp were subjected to primer pair designed to amplify a region of P11- MP F3/B1 (273 bp). All samples gave expected size for the *P. larvae* colonies, these result confirmed that the bacterium tested was *P. larvae*.

Nowadays PCR techniques are more widely distributed than screening of honey samples by classical isolation and identification methods. The acid amplification of a specific target region of the bacterial genome is a rapid and reliable diagnosis method that is preferred over time-consuming, laborious, and expensive microbiological methods [16, 17, 18].

In this study, PCR amplification were done for two amplicon regions ERIC and metalloprotease with band size of 550 bp and 273 bp respectively. The PCR procedure was repeated at least three times for more accuracy. Our results show higher accuracy of molecular methods compared to the microbiological method, in which the percentage of AFB in brood-nest samples was lower. The differences between PCR result and microbiological method can be easily explained by the misdiagnosis of *P. larvae* colonies or by technical misprisions (table 3 and4).

Table 3: The positive and negative results for all samples including both microbiological and PCR with numbers and percentages (in brackets)

Samples types	Microbiological positive (%)	Microbiological Negative (%)	PCR positive (%)	PCR negative (%)
Bulk honey (B=8)	2 (3.1%)	6 (9.2%)	2 (3.1%)	6 (9.2%)
Brood-nest honey (BN=52)	27 (41.5%)	25 (38.5%)	20 (30.8%)	32 (49.2%)
Larvae samples (L=5)	3 (4.6 %)	2 (3.4 %)	3 (4.6 %)	2 (3.1 %)
Total	32 (49.2 %)	33 (50.8 %)	25 (38.5 %)	40 (61.5%)

The result showed that the highest percentage of diseased honeybee colonies was recorded in Irbid (North Jordan) with (20%). Alzarqa (6.2%), Al-Ghor and Amman in the middle part of Jordan with (4.6%) for both regions. Moreover, Al-Balqa with (3.1%), Al-Shubak area is a more widespread disease in the northern parts of Jordan, while it seems to be totally absent in southwest Jordan (table 5). This distribution can be explained by the moving of colonies during the winter season from north area of Jordan to warmer parts, this give an indication to the effect of the environmental conditions on the distribution of the disease from humid northern and middle part to dry southern parts. Arabiat in 2007 [7] showed that the disease extended along the western

parts of Jordan from the north to the middle part of Jordan valley and adjacent highlands, north eastern and south-western regions of Jordan were free from symptomatic honey bee colonies. The distribution of AFB disease can be related to many factors , [2, 8, 13, 19] which include: feeding of bees with local or imported contaminated honey and pollen, drifting of infected bees between healthy and infected frames, robbing honey contain spores of *P. larvae* or by contaminated beekeepers clothes and tools. Jordanian beekeepers must take in consideration the elimination of infected colonies and not including them in the migratory beekeeping practices to avoid spreading the disease to other regions.

Table 4: Honey microbiological tests and PCR results of larvae and honey samples used to detect *P. larvae* according to the location of colonies in Jordan.

Regions	No. of samples	No. of *P. larvae* positive samples by microbiological methods	% of *P. larvae* positive samples by microbiological methods	No. of *P. larvae* positive samples 500 bp fragments	% of *P. larvae* positive PCR
Amman	12	3	4.6%	3	4.6%
Al-Shobak	5	-	-	-	-
Irbid	30	14	21.5%	13	20%
Al-Ghor	9	4	6.2%	3	4.6%
Alzarqa Hashemite University	4	4	6.2%	4	6.2%
Al-Balqa	5	2	3.1%	2	3.1%
Total	65	27/65	41.6%	25/65	38.5%

Table 5: Comparisons between microbiological and PCR methods to detect *P.larvae* from honey and larvae samples from various Jordanian regions

	Type and number of collected samples	*Number and % of positive samples by micro-biological tests*	*Number and % of negative samples by micro-biological tests*	*Number and % positive samples by ERIC-PCR products*	*Number and % negative samples by ERIC-PCR products*
Amman	Brood –nest (4) Bulk honey (6) Larvae (2)	1/4 (1.5%) 1/6 (1.5%) 1/2 (1.5%)	3/4 (4.6%) 5/6 (7.7%) 1/2 (1.5%)	¼ (1.5%) 1/6 (1.5%) 1/2 (1.5%)	3/4 (4.6%) 5/6 (7.7%) 1/2 (1.5%)
Al – Shubak	Brood – nest (5)	0/5 (0%)	5/5 (7.7%)	0/5 (0%)	5/5 (7.7%)
Al- Ghor	Brood – nest (9)	4/9 (6.2%)	5/9 (7.7%)	3/9 (4.6%)	6/9 (9.2%)
Al –Zarqa	Brood –nest (4)	4/4 (6.2 %)	0/4 (0%)	4/4 (6.2%)	0/4 (0%)
Al-Balqa	Brood – nest (5)	2/5 (3.1%)	3/5 (4.6%)	2/5 (3.1%)	3/5 (4.6%)
Ibrid	Brood- nest (25) Brood hoey (2) Larvae (3)	11/25 (16.9%) 1/2 (1.5%) 2/3 (3.1%)	14/25 (21.5%) 1/2 (1.5%) 1/3 (1.5%)	10/25 (15.3%) 1/2 (1.5%) 2/3 (3.1%)	15/25 (23.1%) 1/2 (1.5%) 1/3 (1.5%)
Total	65	27 (41.5 %)	38 (58.5%)	25 (38.5 %)	40 (61.5%)

Conclusion

AFB is present in different regions of Jordan at different rates, the accuracy of molecular methods (PCR) was much higher than microbiological methods and they gave more accurate and sensitive results for identification for *Peanibacillus larvae* in honey bees.

Competing interests

The authors have declared that no competing interest exists.

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