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Diagnosis of *Paenibacillus larvae* from Honeybees in Jordan According to Microbiological and Chemicals Techniques

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ABSTRACT

American Foulbrood Disease (AFB) is considered one of the most virulent bacterial diseases of honeybee (*Apis mellifera*); it has a vital negative impact on the beekeeping industry worldwide. This work includes the subsequent diagnosis including; chemical, microbiological procedure for detection of *Paenibacillus larvae*. During the spring and the summer of 2009, fifty-six honey and larval samples were collected from 56 inspected honeybees 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 chemical and microbiological methods. Honey bees field diagnosis procedures were considered to be the first diagnosis step for AFB, which can be done by the beekeepers themselves. The total of 57 (honey brood and brood nest honey) from different regions of Jordan was inspected to carry *P. larvae* spores with 35%. The percent referred to the ability of Jordanian beekeepers to ascertained AFB symptoms. American foulbrood exists in Jordan with different distribution depending on the environmental condition. This pathology can be best detected by isolation of *Paenibacillus larvae* from Brood-nest honey samples.

Key words: Honey bee, *Paenibacillus larvae,* microbiological techniques, chemicals techniques, Jodany

INTRODUCTION

Honeybee diseases have been a concern of man for thousands of years. There are many pathogens that affect honeybees and the individuals they infect while others affect the entire colony. The most important pathogens of honeybees are the *Varroa* mite, followed by the bacterial foulbrood diseases and a number of viral diseases such as sacbrood and the bees' paralyses (Haddad *et al*., 2008). The most virulent diseases at present are those of brood, specifically American Foulbrood (AFB) and European Foulbrood (EFB) but the later is curable and can be efficiently controlled by antibiotics (Otten, 2003). Other brood diseases include chalkbrood, a fungal disease that appears to be on the rise and sacbrood, caused by a virus (Waite *et al*., 2003).

American Foulbrood (AFB) is the most serious bacterial disease of honeybee brood (*Apis mellifera*). It is a highly contagious, cosmopolitan disease affecting the larval and pupal stages of honeybees (*Apis mellifera* L.). The AFB can appear and spread quickly through the colony and if the colony is left untreated, it dies in short period. The disease spreads when spores are carried on drifting bees, hive parts, beekeepers' clothes and contaminated pollen or honey.

Several methods were used to control the disease, including shaking method, eradication, burning and anti-biotic treatments. Evaluation of synthetic antimicrobials and antibiotics to control foulbrood has been carried out since the 1940s (Kochansky *et al.,* 2001). Antibiotics are routinely used to control (AFB) in honeybees in many countries.

American Foul Brood was detected in Jordanian honeybee colonies by Arabiat (2007), who studied various honeybee diseases in Jordan. However, his survey depended only on foulbrood clinical symptoms that proved the existence of AFB in Jordan. In this study, we aim at answering the following question: how wide spread is AFB in Jordan and what are the most effective diagnostic measures.

MATERIALS AND METHODS

Samples collection: Fifty six honey samples were collected from different apiaries throughout Jordan during the spring and the summer of 2009, as shown in Table 1. They were taken from 56 inspected honeybees colonies according to a perennially field diagnosis by beekeepers, located in 53 apiaries. From these samples, sixty of the samples were honey (brood nest and bulk) samples originating from six governorates (Irbid, Ajloun, Amman, Al-Balqa', Az-Zarqa and Al-Shubak). The rest five samples were larvae samples originating from Irbid and Amman.

Collection of bulk honey samples (B): Eight local bulk honey samples were collected from 8 different Jordanian beekeepers, each sample (25 mL) was taken from extracted honey ready for sale in each apiary (from the harvest of summer, 2009; one honey sample per apiary). In other words, one sample represents many honeybee colonies in the same apiary. Collected honey samples were stored at 4°C until analyzed (OIE., 2004).

Collection of brood-nest honey samples (BN): Fifty-two brood-nest honey samples were taken from capped honey hexagonal cells close to the brood nest (from 56 inspected honey bees colonies located in 51 apiaries) 25 mL samples were collected with a sterile spoon. Then, transferred into a tube and stored at 4°C until used.

Collection of brood samples (L): Larvae (3-5 days old) were collected from two apparently healthy honey bees colonies and three clinically foulbrood diseased honey bees colonies located in 5 different apiaries after determining the point of infection. Each larva sample was kept in 1.5 mL

Table 1: 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	No. of foulbrood larvae samples	No. of healthy larvae samples	No. of samples
Amman	13	4	6	1	1	12
Al-Shabak	3	5	0	0	0	5
Irbid	25	25	2	2	1	30
Al-Ghor	6	9	0	0	0	9
Alzarqa (Hashemite University)	1	4	0	0	0	4
Al-Balqa	5	5	0	0	0	5
Total	53	52	8	3	2	65

eppendorf tube and stored at -20°C until used. The brood frame showing foulbrood disease symptoms, were loosely wrapped in newspaper and then stored at -20°C until used (OIE., 2004).

Diagnosis of AFB disease: Diagnosis of AFB was carried out by several ways field diagnosis, laboratory diagnostic techniques such as pathological method, bacteriological method and Polymerase Chain Reaction (PCR). The sensitivity and specificity among the mentioned approaches are different.

Screening test or rapid test

Pathological method (clinical test): The principle of this test is to observe any clinical symptoms from bee cell, depending on field diagnosis. Comb with AFB had moist and darkened appearance and become concave and punctured as the infection progresses instead of solid and compact brood in the healthy one (Shimanuki, 1997). Larva or pupa color in diseased colonies changes to creamy brown and then to a dark brown, the larva can become glutinous and can be drawn out as threads when a probe is inserted, it is one of the best known technique for field diagnosis (match-stick test) (OIE., 2008a) (Fig. 1).

Microscopic examination: This method was used to observe the morphology of *Paenibacillus larvae* to distinguish the AFB from other brood diseases.

Spores staining: A preliminary check to the inspected diseased larvae in a direct way by using suitable spores stain such as 0.2% carbol fuchsin (OIE., 2008b). About two drop of water was mixed with the sample (larvae), the suspension was then transferred by loop and smeared on a glass slide as thin film. The slide was then stained with 0.2% carbol fuchsin for 30 sec. After washing and drying the slide, it was examined under the microscope (Reichert, USA) for P.l. larvae spores, which are ellipsoidal and about 1.3×0.6 mm.

Gram staining: A smear of suspected larvae colony was taken on a slide , Gram stained, the slide was flooded with crystal violet for 60 sec, washed then flooded again with iodine solution for 1 min, decolorizing agent was used as ethanol for 5 sec, the final steps involved applying safranin, after each steps the slide was rinsed with water for 5 sec. The prepared slide was then examined under

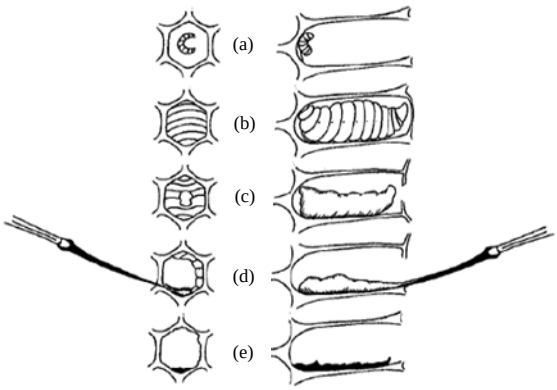

Fig. 1(a-e): Progression of the disease: (a) Point of infection, (b) Larval development to prepupal stage, (c) Cell content reduced and capping is punctured, (d) Cell content became glutinous and (e) Residual scale tightly adheres to the bottom of cell

microscope ((Reichert, USA) and photographed (Motican, Austria). The bacterium was identified as *P. larvae* with gram positive rods 0.5-0.6 μm wide and 1.5-6 μm long as illustrated in Fig. 1 (Shimanuki and Knox, 1988; Bailey and Ball, 1991; Alippi, 1995; Heyndrickx *et al*., 1996).

Holst milk test: This is a simple tests based on the high level of protease that sporulating *P. larvae* produced. A smear of diseased larvae was suspended in a tube containing 3-4 mL of 1% skim milk in water. The tube was then incubated at 37°C. If *P. larvae* are present, the suspension is clear in 10-20 min (Shimanuki and Knox, 1988).

Confirmation test (identification of *Paenibacillus larvae*): The *Paenibacillus larvae* isolates were identified by colonial morphology and a set of biochemical tests.

Bacteriological method: This method depends on bacterial cultivation and isolation steps in order to obtain pure cultures. Several media for cultivating *P. larvae* were examined to choose the appropriate one. The media used approved by OIE (2008b) and reported by other researchers as a good cultivation media to the target bacterium are: Columbia sheep blood agar (CSBA) from Difco (France), with 5% sterile horse or sheep blood (JOVAK) per litter of media (Alippi, 1995).

Brain heart infusion medium (BHIT): (Difco laboratories fortified with thiamine hydrochloride 100 μg L^{-1}, 2% agar and adjusted pH to 6.6 with hydrochloric acid) 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. 30 μg mL^{-1} Nalidixic acid (Sigma, USA) was added for all media types, after media reach 50°C to prevent any antibiotics exposure. In order to inhibit the growth of *Paenibacillus alvei* the Gram-negative bacteria or any other spore- forming bacteria that may completely cover the plate (Hornitzky, 1988; Alippi, 1995; Iurlina *et al*., 2006) without having any effects on *P. larvae* viability (Fries and Raina, 2003).

Isolation of *Paenibacillus larvae* from AFB infected honey samples: Honey contains a high concentration of carbohydrates and other natural bacteriostatics substance. (Shimanuki, 1997). It was also necessary to examine honey for the presence of *P. larvae* For Bulk Honey, 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 (1XPBS). After vigorous mixing, the suspension was centrifuged 20 min at 27,000 rpm by ultracentrifuge (Beckman Coulter, Inc. California, USA) 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 (Hansen, 1984; Neuendorf *et al*., 2004) by taking 5 mL of the honey samples diluted (1:1 v/v) with Sterile distil water , the solutions incubated for 15 min at 80°C (heat shocked), then the suspension was ready to inoculates on different media types.

Isolation of *Paenibacillus 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). The 100 μL of the suspension was diluted in 900 SDW, vortex mixed, then the suspension were centrifuged 2000 rpm for 5 min (Alippi *et al*., 2004). The 200 μL from

Table 2: Colony morphology on different media types

Media types	Colonies morphology
CSBA	Small, regular, glossy but yrous, grayish
BHIT	Small, regular, beige colored with slightly rounded end
MYPGP	Small, regular, mostly rough, flat or raised and whitish to beige colored

each sample were inoculated over the surface of the three media types (3.2.2.1) and then incubated at (34-37)°C for 2-4 days (CO_2 incubator, USA).Colonies morphology appeared depending on the media types (Table 2).

Biochemical tests: Paenibacillus larvae were identified by its biochemical characteristics. About two small colonies from suspected samples were tested as follows.

Catalase test: A drop of 3% hydrogen peroxide (H_2O_2) placed on an actively growing culture on a solid medium. Most aerobic bacteria break down the peroxide to water and oxygen and produce bubbly foam but *P. larvae* is almost negative to this reaction (Haynes, 1972; Hansen and Brodsgaard, 1999; Ritter, 2003).

Hydrolysis of starch: The suspected culture inoculated on plates of J- medium (5 g L^{-1} Tryptone, 15 g L^{-1} yeast extract, 3 g L^{-1} dipotassium hydrogen phosphate (K2HPO4), 20 g L^{-1} agar) and 1% glucose) and incubated at 37ºC, after 5-10 days the plates flooded with iodine solution at room temperature for 15-30 min. If the culture is *P. larvae*, no clearing zone will be observed (Neuendorf *et al*., 2004).

Hydrolysis of casein: The suspected culture aged 24 h was inoculated on the medium (10% skim milk and 3% agar) the medium sterilized at 121 C for 15 min), incubated for 7 days at 37ºC. If the suspected culture is *P. larvae*, clear zone around the growing culture will be observed due to casein hydrolysis (Neuendorf *et al*., 2004).

Hydrolysis of gelatin: The suspected colonies were inoculated on gelatin medium (12% gelatin, pH was adjusted 7.0/1 L) and incubated at 28°C. The hydrolysis of gelatin was observed every three days for four weeks. The colonies were considered as *P. larva* if the gelatin became liquefied because *P. larvae* is able to hydrolyze gelatin.

RESULTS AND DISCUSSION

Diagnosis of American foulbrood

Diagnosis of AFB by using pathological methods (field test): The broods of five honeybee colonies were inspected in five different apiaries located in Amman/Abu-nseear (2 L; L; larval remains) and Irbid/Alneeamy (3 L), Three out of 5 inspected honeybee's colonies (60%) showed the clinical symptoms of AFB disease according to their appearance in which infected larvae color turned brown then black and to matchstick test (Fig. 2).

Brood-nest Honey samples (52 BN; BN brood-nest) were collected from the field after primary diagnosis by beekeepers as AFB disease according to field tests to the brood frames, in which diseased colonies have foul smell with spotty brood pattern.

Diagnosis of AFB in the laboratory

Isolation and identification of *Paenibacillus larvae* from larval remains: The three larval samples originated from the three infected honeybee colonies showed clinical symptoms of AFB

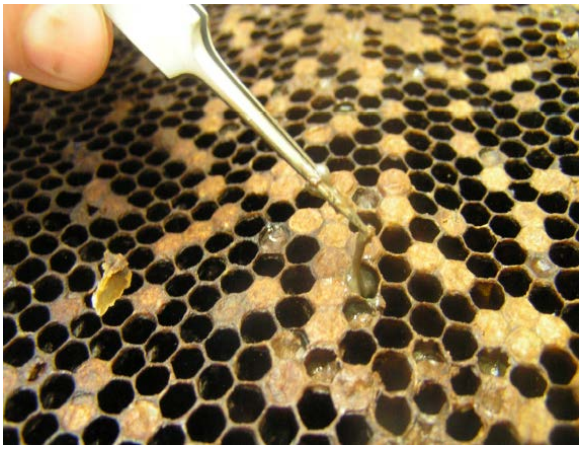

Fig. 2: Matchstick test showing an infected Brood with clinical symptoms signs for AFB (Irbid/Maru)

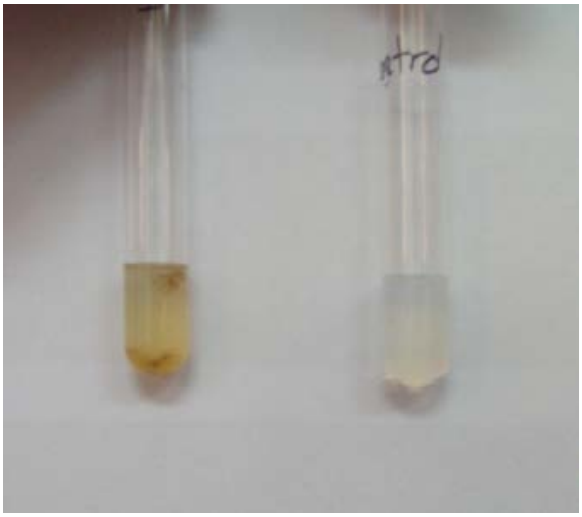

Fig. 3: Holst milk test (1% skim milk Right: control, left clear suspension after 10 min incubation at 37°C

disease, were analyzed using Holst milk, spore staining and microbiological methods and 60% were again confirmed as infected with *P. larvae*.

The bacterial colonies were isolated from AFB infected larval remains and from apparently healthy larvae samples on Columbia Sheep Blood Agar (CSBA) (Fig. 3, 4), MYPGP and Brain Heart Infusion with Thiamine (BHIT), (Fig. 5, 6). Infected larvae were classified as *P. larvae* when colonies were small, regular appearance with glossy but yrous and grayish on CSBA. Beige colored with slightly rounded end on BHIT and mostly rough, flat or raised, whitish to beige colored on MYPGP.

Isolation and identification of *Paenibacillus larvae* from Honey samples: A total of 60 honey samples (52 brood-nest and 8-bulk honey) from different honeybees colonies were collected

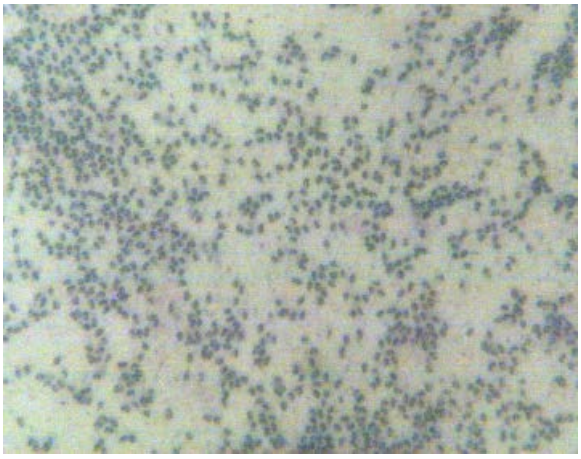

Fig. 4: Spores staining with 0.2% carbol fuchsin showing signs for *Paenibacillus larvae* criteria (Motican 10X)

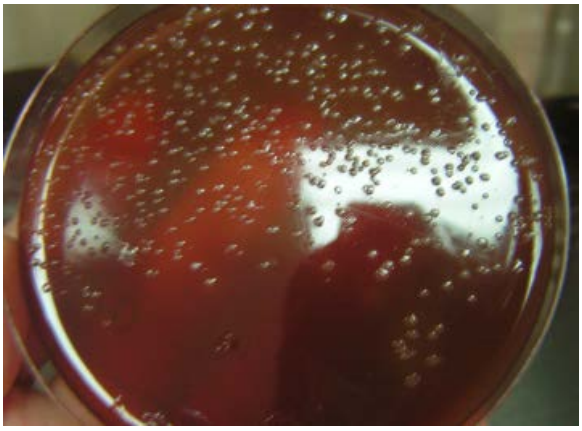

Fig. 5: *Paenibacillus larvae* colonies isolated on CSBA from larval remains after 48 hr incubation with 5% CO25% CO2 (Amman/Abu nseear 2009)

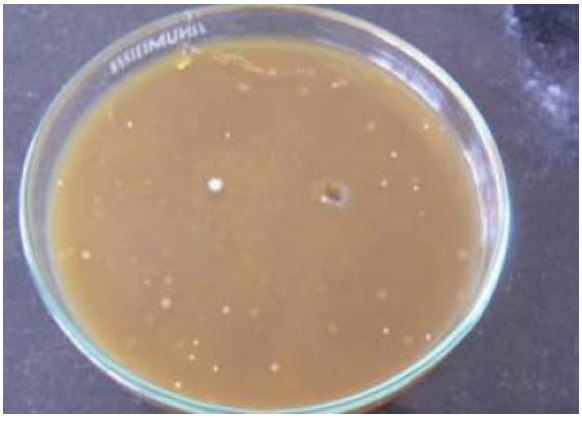

Fig. 6: *Paenibacillus larvae* colonies isolated on BHIT from larval remains after 48 h incubation with 5% CO2 (Egypt/Owayss, 2007)

from 45 apiaries in Jordan. The 52 brood-nest honey samples were considered to have *Paenibacillus larvae* spores depending on the primary field diagnosis made by beekeepers before samples were collected from the expected infected brood frames. The eight Bulk honey samples were not had any information about infection presented.

Honey samples were analyzed by several bacteriological methods. The result showed that 29 out of 60 tested honey samples (48%) yielded isolates positively identified as *P. larvae* colonies on culturing media. Both AFB clinically symptomatic diseased honeybee colonies and healthy honeybee colonies (according to the primary diagnosis by beekeepers as infected samples) showed *P. larvae* infection. On the other hand, healthy samples in which the bacterium was not found, with well-known infection history used as negative control.

Paenibacillus larvae was detected in brood-nest honey and bulk honey originating from both apparently healthy and symptomatic AFB disease with an incidence of 27 (45%) and 2 (3%), respectively.

Isolation of *Paenibacillus larvae* from brood-nest honey samples: From the 52 brood-nest honey samples, 27 (52%) were positive for *P. larvae* bacterium on the three types of media, isolated bacterial colonies were gram stained and classified as *P. larvae* (Table 3).

Isolation of *Paenibacillus larvae* from bulk honey samples: A total of 25% (2 out of 8) of bulk honey samples isolated were positive for *P. larvae* bacterium on the CSBA, BHIT and MYPGP medium.

For further identification all positive 32 samples (brood-nest, bulk and larval remains) were gram stained (Fig. 7-8) and tested with Catalase test (3% H_2O_2). Isolates produced bubbly foam were considered as negative samples for *P. larvae* (Table 4).

Diagnosis by biochemical tests: Isolated *Paenibacillus larvae* from (Bulk, Brood nest and larval remains) were tested by four biochemical tests for further conformation and existence of *P. larvae*. 27 out of 32 (84%) samples were confirmed as *P. larvae* (Table 4).

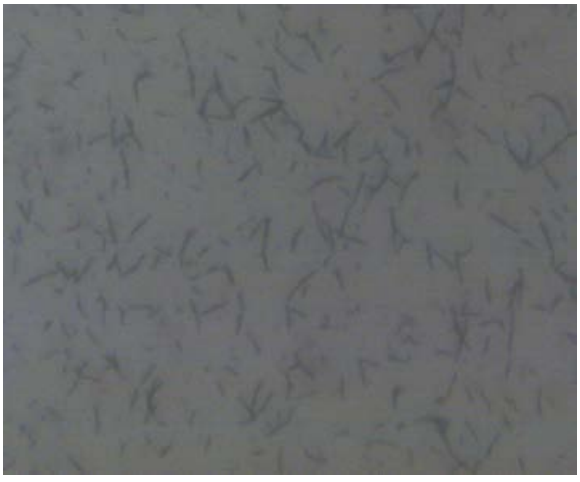

Fig. 7: Isolated colonies from BN samples gram stained, showing vegetative cells of *Paenibacillus larvae* under Microscopic examination (100X)

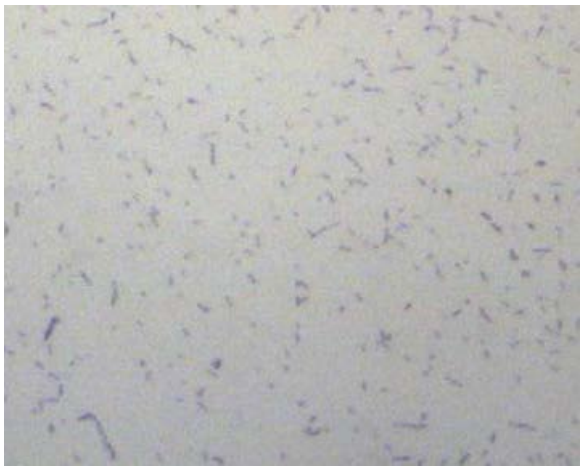

Fig. 8: Isolated colonies from B samples gram stained *Paenibacillus larvae* under microscopic examination (10X)

Table 3: Result of isolation of *Paenibacillus larvae* from brood-nest honey samples collected from different regions in Jordan and their percentages

Region	No. of inspected colonies	No. of colonies showing AFB symptoms	Colonies showing AFB symptoms (%)
Amman	6	2	2/52 (3.8)
Al-Shbak	5	0	0/52 (0)
Irbid	25	15	15/52 (29)
Al-Ghor	9	5	5/52 (9.6)
Alzarqa (Hashemite University)	4	4	4/52 (7.7)
Al-Balqa	5	1	1/52 (1.9)
Total	52	27	27/52 (52)

Table 5 shows the percentage and the number of positive isolates. There was some variation in the result according to the biochemical tests but at least 84% were confirmed as *P. larvae* infections. The gelatin hydrolysis showed 100% infections.

Inspection of the five sealed brood frames showed that 3 out of 5 showing discoloration sunken and punctured capping. Larvae died in the upright position, their colored turned to brown then to black, the consistency of dead brood were soft and sticky with unpleasant odor (foul or something like glue odor), the match stick were inserted and pulled out, the remains of the dead larvae form a thread (Shimanuki and Knox, 1988; Otten, 2003; Lindstrom and Fries, 2005; OIE., 2008a; Adjlane *et al*., 2012).

Honey bees field diagnosis procedures were considered to be the first diagnosis step for AFB, which can be done by the beekeepers themselves. The total of 57 (honey brood and brood nest honey) from different regions of Jordan was inspected to carry *P. larvae* spores with 35%. The percent referred to the ability of Jordanian beekeepers to ascertained AFB symptoms.

In this study three specific media were used to isolate the causative agent of AFB disease *Paenibacillus larvae* from collected honey and larval samples. Bacterial colonies were easy to discriminate among other bacteria grown on the media according to their criteria (Fig. 6-7). The most observed bacterium growth were in CSBA, when *P. larvae* grown the media become discolored or partially heamolysed and that typically met with Heyndrickx *et al*. (1996) study.

A total of 32 samples (brood-nest honey, bulk honey and larval remains) out of 65 samples (49.2%) were identified as AFB disease infected samples according to bacteriological methods

Table 4: Result of detection *Paenibacillus larvae* using microbiological tests

Samples	Morphology	Gram stain	Carbol fu.	Catalase test	Starch hydrolysis	Hydro-of casein	Gelatin-hydrolysis
2B	+	+	+	+	+	+	+
1BN	+	+	+	+	+	+	+
1L	+	+	+	+	+	+	+
10BN	+	+	+	+	+	+	+
12BN	+	-	-	-	-	-	+
15BN	+	+	+	+	+	+	+
16BN	+	+	+	+	+	+	+
18BN	+	-	-	-	-	-	+
19BN	+	+	+	+	+	+	+
20BN	+	+	+	+	+	+	+
21BN	+	+	+	+	+	+	+
24BN	+	+	+	+	+	+	+
27BN	+	-	+	+	+	+	+
28BN	+	+	-	-	-	-	
29BN	+	+	-	-	-	-	+
30BN	+	+	-	-	-	-	+
32BN	+	+	+	+	-	-	+
34BN	+	-	+	+	+	+	+
36BN	+	+	+	+	+	+	+
37BN	+	+	+	+	+	+	+
38BN	+	-	+	+	+	+	+
40BN	+	+	+	+	+	+	+
42BN	+	+	+	+	+	+	+
43BN	+	+	+	+	+	+	+
45BN	+	+	+	+	+	+	+
46BN	+	+	+	+	+	+	+
50BN	+	+	+	+	+	+	+
7B	+	+	+	+	+	+	+
8B	+	-	-	-	-	-	+
3L	+	+	+	+	+	+	+
4L	+	+	+	+	+	+	+

BN: Brood-nest samples, B: Bulk honey samples and L: larvae remain

Table 5: Percentages (in brackets) and numbers of *Paenibacillus larvae* infections according to biochemical tests

	Positive isolates out of 32	
Biochemical tests	No.	(%)
Starch hydrolysis	27/32	84
Gelatin hydrolysis	32/32	100
Catalase hydrolysis	27/32	84
Hydrolysis of casein	27/23	84

techniques. The identification steps included colony characterization after staining with suitable stain (gram stain) followed by microscopic examination (10-100X immerse). *Paenibacillus larvae* detected with criteria as positive for gram stain, slender rod with slightly rounded ends. Then Catalase test were carried out in which *P. larvae* were not formed any foamy bubbles because it was not able to break down the peroxide into water and carbon dioxide includes (Shimanuki and Knox, 1988; Hansen and Brodsgaard, 1999; Otten, 2003; De Graaf *et al*., 2001; OIE., 2008a; Adjlane *et al.*, 2014).

Biochemical tests were used for further confirmation and existence of *P. larvae*. The 32 samples which considered as AFB infected samples were tested by four biochemical tests.

The result showed that there were some variation between different tests but at least 84% (27 out of 32) were confirmed as *P. larvae* for starch, catalase, gelatin and casein hydrolysis, in which *P. larvae* digested gelatin and casein but was unable to digest starch. For gelatin hydrolysis all positive and negative samples (100%) liquefied gelatin, it is easily to describe that many other bacteria can digest gelatin such as. Catalase test described above. Thia Agricultural Standard

(TAS) in diagnosis of American foulbrood in bee (Arabiat, 2007) used such these biochemical tests to identified *P. larvae* according to its biochemical characteristics.

CONCLUSION

This project provides several conclusions and recommendation, which can be obtained as the following:

- American foulbrood exists in Jordan with different distribution depending on the environmental condition
- American foulbrood can be best detected by isolation of *Paenibacillus larvae* from Brood-nest honey samples
- Beekeepers must pay attention when moving colonies during seasons to a void the spread of pathogens include AFB causative agents

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