J. Agric. Sci. Mansoura Univ., 33(11): 8219 - 8228, 2008

IDENTIFICATION OF THE *Paenibaccillus larvae* AS A PATHOGEN CAUSATIVE OF (AFB) DISEASE IN HONY BEE COLONIES AND STUDYING THE ANTIBACTERIAL ACTIVITY OF THE PROPOLIS.

Mostafa, Shaimaa N.[*]; O. EL-Ansary[*]; A.A. EL-Shemy[**] and M.A. Ewies[**]
*** Dept of Economic Entomology. Fac. Of Agric., Alexandria Univ., Egypt**
****Dept of Economic Entomology and Pesticides. Fac. Of Agric., Cairo Univ., Giza, Egypt**

ABSTRACT

Isolation and identification of the causative pathogen bacteria of the AFB (*Paenibaccillus larvae*) from the Egyptian honey bee colonies were conducted in this study. The identification was achieved by many tests which assured the pathogen causative. This tests were Roby tests, Holst milk test, selective medium, catalase production, hydrolysis of starch and microscopic examination which involved negative stain, spores staining and Gram stain test. Also a comparison between Egyptian, Bulgarian propolis ethanolic extract and three antibiotics which were tylosin, penicillin and combination of ampicillin and gentamicin from the standpoint of their bacteriostatic effect on three bacterial species which were *Paenibaccillus larvae*, *Listeria monocytogenes* and *Staphylococcus aureus* the comparison was conducted by the technique of measuring the inhibition zone diameter (IZD). The obtained results proved that the Bulgarian propolis ethanolic extract was more effective than local propolis ethanolic extract. Also the *S. aureus* was more sensitive to propolis ethanolic extract (PEE) than *L. monocytogenes*, while *P. larvae* was more resistant for PEE.

INTRODUCTION

American foulbrood (AFB) is one of the most important diseases that attacks honey bee (*Apis mellifera*) in the larval stage. The AFB disease is caused by the gram-positive bacteria with the scientific name, *Paenibaccillus larvae* subsp. *Larvae*. The disease is causing severe damage to the beekeeping industry world wide by destruction the infected bee colonies. Only the spore stage of *P. larvae* can initiate infection (Morse & Nowogrodzki, 1990, Cox *et al.,* 2005, Owayss, 2007, Zakaria 2007 and Traynor, 2008).

Propolis is a complex resinous yellow – brown to dark brown mixture that collected by honey bees from plant exudates for construction, protection and adaptation of their nests (Abu Fares *et al.*, 2008). Many researchers demonstrated the properties of propolis, and their investigations had done using propolis from different geographic locations to describe the antimicrobial activity of propolis (Dawoud *et al*., 1994, Serra *et al*., 1994 and Bosio *et al*., 2000). The propolis had an antibacterial effect against most gram-positive bacteria especially (*Staphylococcus aureus*) as well as some the gram-negative types (Abd-Al-Fattah *et al*., 1993 and Dawoud *et al*., 1994). Recently, a combination of some antibiotics and propolis was applied to increase the antimicrobial activity of such antibiotics as in the case of antistaphylococcal action and its effect on the local treatment of many staphylococcal diseases (Salah *et al*., 2007).

The aim of this work is to: (1) isolation and identification of the causative pathogen bacteria of the AFB (*Paenibaccillus larvae*) which infected honey bee colonies at Alexandria Governorate. (2) Determination the antibacterial activity of the Egyptian and Bulgarian propolis using the paper-disc diffusion technique.

MATERIALS AND METHODS

Propolis samples:

Two propolis samples were used. The first sample was collected from honey bee colonies in Alexandria Governorate and the second sample was imported from Bulgaria. Ten grams from each propolis were extracted by ethanol according to the technique of Ashour (1989).

Tested bacteria

Three Gram-positive (G+) bacterial strains were used for antibacterial activities as follows: a) *Paenibaccillus larvae*. The spores were collected from a comb infected with AFB disease from the apiary of Alexandria Faculty of Agriculture, b) *Listeria monocytogenes* which infected human and c) *Staphylococcus aureus* FR1S6. The two pathogens (b & c) were obtained from dairy department (microbiology branch), Faculty of Agriculture, Alexandria University.

Cultivation media:

Medium"1" Brain heart infusion with thiamin **(BHIT)** was used to culture the causative agent of American foulbrood (*Paenibaccillus larvae*) which gives good vegetative growth without sporulation. This medium consists of 55g brain heart infusion (Difco) fortified with 0.1 mg thiamin hydrochlorid per liter (shimanuki and Knox, 1994).

Medium "2"(Bailey's medium) this medium was followed to obtain good vegetative growth and sporulation for the causative agent of American foulbrood (*Paenibaccillus larvae*). The medium consists of 10 g yeast extract, 10 g glucose, 10 g soluble starch, 20 g agar and 13.5 g K_2HPO_4 per liter (Bailey and Lee (1962). Bacterial colonies were arisen from this medium used in spore staining experiment.

Medium "3" Brain heart infusion **(BHI)**: This medium consists of 12.5 g calf brain solids, 5 g beef heart solids, 10 g proteose peptone, 2 g dextrose, 5 g sodium chloride and 2.5 g disodium phosphate per liter. This medium was used for *Listeria monocytogenes* and *staphylococcus aureus*.

Identification for *Paenibaccillus larvae*:

The following tests were conducted:

1. Roby test.

Larvae that have died of American foul brood disease exhibit a "ropy" condition that can be demonstrated by inserting a matchstick or similar implement into the dead and mass and drawing out the material into a threadlike projection longer than 2.5 cm. (Morse and Nowogrodzki, 1990).

2. Holst milk test.

The Holst milk test (Holst, 1946) is a simple test based on the high level of proteolytic enzymes produced by sporulating *P. larvae.* The test was

conducted by suspending a suspect scale or smear of a diseased larva in a tube containing 3 to 4 ml of 1% powdered skim milk in water. The tube is then incubated at 37ºC. if AFB is present, the suspension should clear in 10 to 20 minutes. It should be noted that this test is not always reliable.

3. Identification of bacteria on selective medium.

To obtain pure bacterial colonies, ten smears of infected honey bee larvae were homogenized in 1 ml sterilized water and pipetted to 1.5 ml Eppendorf tub. This suspension was spread on Petri-dishes containing two selective media. BHIT and Bailey's medium these media were detailed before. Two media were adjusted to pH 6.6 with HCl and autoclaved at 10 Ib/sq.in., at 116ºC for 20 min. After inoculation of plates with larval suspension, the BHIT medium was incubated at 35ºC for 3 days (shimanuki and Knox, 2000) while Bailey's medium was incubated at 35ºC for 9 days according to Jelinski (2003).

4. Catalase production:

A drop of 3% hydrogen peroxide was placed on an actively growing culture on a solid medium. Most aerobic break down the peroxide to water and oxygen and produce a bubbly foam, but non aerobic bacteria almost always negative for this reaction. (Haynes, 1972)

5. Hydrolysis of starch:

BHIT medium was prepared and fortified with 4 g soluble starch per liter. The medium was autoclaved at 10 Ib/sq.in. at 116ºC for 20 min., then medium was put in 3 Petri-dishes and inoculated with bacterial colony (*P. larvae*) as a line in the center of medium. The plates were incubated at 35ºC for 48 hour. After incubation, Lugol's iodine solution was placed on the 3 plates for 2 hours the plates have to inspect and observing if there are non blue colored areas around the inoculated line with bacteria, absence of blue color indicated that the starch was analyzed. The presence of blue color around the bacterial colonies means that the starch not analyzed. (Abo EL-Dahab and EL-Goorani, 1984).

6. Microscopic examination:

This examination was very useful for differentiating between American foulbrood (AFB) and other brood diseases. The microscopic examination involved negative stain, spores staining and Gram stain test were conducted according to Abo EL-Dahab and EL-Goorani (1984).

Assessment of the antibacterial activity:

In this study three different spices of bacteria were used; *P. larvae, S. aureus* and *L. monocytogenes*. Two types of media (BHIT and BHI) were used to cultivate the above species of bacteria. Three types of antibiotics were used; tylosin which used against *P. larvae*, penicillin which used against *S. aureus* and combination of ampicillin and gentamicin which used against *L. monocytogenes*. The third antibiotic was obtained from Glaxo Welcome Company, Cairo. Five concentrations from ethanolic extract of propolis (EEP) were used from both Egyptian and Bulgarian propolis. These concentrations were 100, 250, 500, 750 and 1000 ppm., which containing 5, 12.5, 25, 37.5 and 50 µg propolis respectively. For the antibiotics only one concentration of 100 ppm was used which containing 5µg for tylosin or penicillin, while the combination of ampicillin and gentamicin was contained 6µg ampicillin and

0.05µg gentamicin. Mixture from ethanol alcohol (absolute) and water (10: 1) was used as control. Every concentration was replicated three times.

The two types of media were autoclaved at 10 Ib/sq.in. at 116ºC for 20 min. 0.1 ml of bacterial suspension was homogenized in 15 ml medium and spread on Petri-dishes. Small paper disk was placed in the center of the medium in every pleat. 50µl from (EEP), tylosin, penicillin and control were taken by micro-pipette and placed on a small paper disk but 60.5µl was taken from the combination of ampicillin plus gentamicin. The plates were incubated at 35ºC for 24 hours. Finally, inhibition zones were measured. (Cox *et al*., 2005 and Azmi *et al*., 2008).

RESULTS AND DISCUSSION

Identification of *Paenbacillus larvae*

1. Roby test.

When the Roby test was executed according to the steps previously illustrated in the materials and methods, it was found that the infected larvae were positive for this test.

2. Holst milk test.

When the Holst milk test was carried out the suspension consisted of skim milk and the *Paenbacillus larvae* became clear in 15 minutes (Fig 1). So, the Holst milk test was positive. It was explained by Holst (1946). He reported that the *Paenibacillus larvae* bacteria produce a high level of proteolytic enzymes which cause the precipitation of the milk proteins, and then the mixture becomes clear.

3. Identification of bacteria on selective medium.

Two specific medium were used to isolate bacteria responsible for AFB in the tested samples. Bacterial colonies were purified on the BHIT and Bailey's medium; these two media were selective for *Paenibacillus larvae*. (Fig 2& 3). BHIT medium gives a good vegetative growth but not sporulation and incubated for 3 days only (Fig 2). The bacterial colonies grew on Bailey's medium give good vegetative growth and sporulation and incubated for 9 days (Fig 3). So, in the present work the colonies produced form BHIT were used in the investigations of the catalase test, starch test, negative staining and Gram stain. The colonies produced from Bailey's medium were used in the identification of the spores and to make comparison between the colonies.

4. Catalase production:

The bacterial colonies isolated from BHIT medium gave a negative reaction with catalase test; it was explained by Sneath *et al***.** (1986) who reported that the negative catalase test proved that the obtained bacteria cannot produce catalase enzyme which analysis H_2O_2 (Fig 4), so it may expected to be *Paenbacillus larvae*. The present result was in accordance with Shimanuki and Knox (2000) who studied the differentiation of spore forming bacteria in honey bees. They concluded that the *Bacillus alvei* or *Bacillus laterosporus* are positive with catalase test, but a *Paenibacillus larvae* was negative with catalase test. Also *Paenibacillus larvae* was non

aerobic bacteria because most aerobic bacteria break down the peroxide to water and oxygen and produce a bubbly foam in this test but *Paenibacillus larvae* was negative for this reaction.

5. Hydrolysis of starch.

The bacterial colonies isolated from BHIT medium gave a negative reaction with hydrolysis of starch test. The presence of blue color around the bacterial colonies means that the starch not analyzed (Fig 5).It was explain by Abo EL-Dahab and EL-Goorani (1984) whom illustrated that some of bacteria species can excrete exo-enzymes (amylases) which analyze the starch molecules. These enzymes are α-amylase, β-amylase and Amylo 1-6 glucosidase. These enzymes help in starch hydrolysis process to maltose. Sneath *et al***.** (1986) reported that the *Paenibacillus larvae* was negative for hydrolysis of starch.

6. Microscopic examination:

a. Negative stain.

The inspection of the prepared films from the cultures of BHIT medium illustrated that the bacterial cells were appeared as rod shape with bright or white color on black ground in single or pairs or chains (Fig 6) as well as the descriptions of Alippi (1991).

b. Spores staining.

The prepared films from cultures of Bailey's medium were inspected. It was found that the endospores were appeared in red color but the sporangium and vegetative cells were appeared bright on grey ground (Fig 7). The spore was oval and about twice as long as wide, about 0.6 × 1.3 µm as reported by Shimanuki & Knox (2000) and Peters *et al.* (2006)

c. Gram stain test.

This test was accomplished by using the microscopic examination of the stained films which prepared from the cultures of BHIT medium. It was found that the bacterial cells were stained with violet color. The bacteria were a slender rod with slightly rounded ends and have a tendency to grow in chains (Fig 8). The rod varies greatly in length, from about 2.5 to 5 µm in wide. The obtained results were in accordance with those of (Shimanuki & Knox, 2000 and Owayss, 2005) whom studied the American foulbrood disease and diagnosed the *Paenibacillus larvae.*

Assessment of the antibacterial activity:

Data found in table (1) demonstrated the results of the comparative study between Egyptian and Bulgarian propolis and three antibiotics, (tylosin, pincillin and the combination of ampicillin & gentamycin) against three pathogen bacteria which are *Paenibacillus larvae*, *Staphylococcus aureus* and *Listeria monocytogenes*. The criteria of inhibition zone diameters were used in this comparison. In the case of *Paenibacillus larvae*, the inhibition zone diameters (IZD) for the Egyptian propolis were 10, 12, 25, 31.6 and 48.6 mm. respectively, for the concentrations of 100, 250, 500, 750 and 1000 ppm. The Bulgarian propolis recorded IZD of 12.66, 16.66, 30, 36.33 and 52.33 mm., respectively, for the above tested concentrations. The antibiotic tylosin was tested by one concentration of 100 ppm., and caused IZD of 51.33 mm. The second tested pathogen was *Staphylococcus aureus* IZD of 20.3, 27, 34.6, 39 and 52 mm., respectively, for the above mentioned concentrations.

The records of Bulgarian propolis were scored inhibition IZD of 25.66, 32.66, 37.33, 43.66 and 58.33 mm., respectively, for the above tested concentrations. The antibiotic pincillin caused IZD of 40.5 mm., for one concentration used which was 100 ppm. Finally, pathogen bacterial of *Listeria monocytogenes* was tested also by criteria of IZD. The Egyptian propolis produced IZD of 15.3. 23.3, 30.3, 34.6 and 49.3 mm., respectively, for the above tested concentrations. On the other hand, the Bulgarian propolis produced IZD of 19.6, 28.33, 37.66, 40.66 and 54.33 mm., respectively, for the above tested concentrations. The combination of ampicillin and gentamycin with the concentration of 100 ppm., was produced IZD of 45 mm. (table 1).

Table 1: Diameters of inhibition zones (in mm.) occured by propolis ethanol extracts (Egyptian & Bulgarian) and three antibiotics (in ppm.) against some pathogen bacteria.

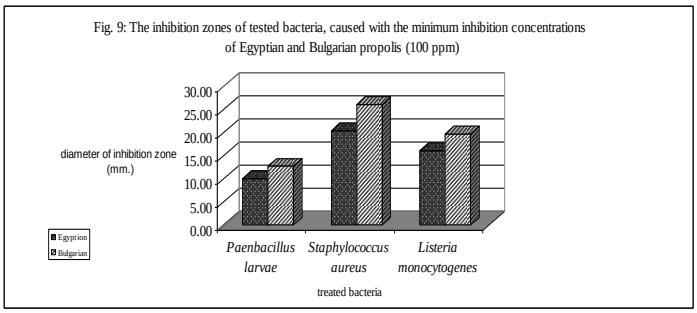
Treated bacteria	*Paenibacillus larvae*			*Staphylococcus aureus*			*Listeria monocytogenes*		
Concentration (ppm.)	Egyptian propolis	Bulgarian propolis	Tylosin	Egyptian propolis	Bulgarian propolis	Pincillin	Egyptian propolis	Bulgarian propolis	combination of mpicillin& gentamycin
100	*10.00	12.66	51.33	20.30	25.00	40.50	15.30	19.60	45
250	12.00	16.66	**-	27.00	32.66	-	23.30	28.33	-
500	25.00	30.00	-	34.60	37.33	-	30.30	37.66	-
750	31.60	36.33	-	39.00	43.66	-	34.60	40.66	-
1000	48.60	52.33	-	52.00	58.33	-	49.30	54.33	-

* **Inhibition zone diameter.**
** **The concentration not used.**

Many studies which carried out in the present field were in accordance with the present results. As example, Cox *et al.* (2005) reported that Dr. Shimanuki from the Beltsville Bee Laboratory defined the resistant AFB as having an IZD of 20 mm or less. Therefore, any sample with an IZD larger than 20 mm was designated as susceptible. Also, Cox *et al*. (2005) tested 16 AFB positive colony samples for susceptibility to tylosin antibiotic. The average IZD of tetracycline resistant sample was 51 mm. and susceptible samples recorded 50 mm. These results indicate that AFB is quite susceptible to tylosin and therefore, tylosin is still very effective in inhibiting the growth of AFB. It would be interesting to test more samples for susceptibility to this drug a few years from now.

On the other hand, the IZD of the minimum inhibition concentration (MIC) for the Egyptian and Bulgarian propolis were determined in the present study. The IZD for the Egyptian propolis recorded 10, 20.3 and 15.3 mm. for the *P. larvae*, *S. aureus* and *L. monocytogenes* respectively, at the concentration of 100 ppm. The Bulgarian propolis recorded IZD values of 12.66, 25.66 and 19.6 mm. at the concentration of 100 ppm., respectively for the above three tested bacteria. (Fig 9).

figs



Similar results were observed by Salah *et al*. (2007) who studied the (MIC) of propolis susceptible *S*. *aureus* and found that the MIC of propolis ethanolic extract (PEE) were 5, 4 and 3 mg/ml according to the number of susceptible strain, while they were 10 and 8 mg/ml for the MIC of propolis methanol extract (PME). The MIC of propolis hexane extract (PHE) recorded 50 and 45 mg/ml, while the propolis water extract (PWE) had no antibacterial activity. While Serra *et al*. (1994) studied the bacteriostatic activity of 15 propolis samples. The MIC of propolis was about 53 times higher than that reported for tetracycline against *Bacillus subtilis* and *Staphylococcus aureus,* and 400 times higher against *Escherichia coli*

It may be concluded from the present results that *S. aureus* was more sensitive to propolis ethanolic extract (PEE) than *L*. *monocytogenes*, while *P. larvae* was more resistance to propolis ethanolic extract (PEE), specially for the concentrations less than 500 ppm.

The present results were agree with those of many authors, as Abd-Al-Fattah *et al*. (1994) reported that the *S. aureus* representing pathogenic bacteria was found to be very sensitive towards PEE than *Actinomyces bovis*. The inhibition zones ranged between 13-66 mm. and 1-37 mm., respectively in presence of high concentrations > 150 ppm. Dawoud *et al*. (1995) found that the gram-positive bacterial strains such as *S. aureus* have shown high sensitivity to local and foreigner propolis extracts, where these strains affected at 0.2 mg or less propolis/disc.

On the other hand, the Bulgarian propolis was seemed to be more affective than the Egyptian propolis. Dawoud *et al*. (1995) observed that the foreigner propolis extract had a stronger antimicrobial effect than the effect of the local propolis extract.

After the inhibition zone diameters were determined, the observations for the tested plates were carried out to detect if there is bacterial growth or not. The daily observations proved that the bacterial growth was found in the inhibition zones areas after 72 hours in the plates treated with antibiotics. It was also found bacterial growth for the plates treated with the Egyptian and Bulgarian propolis at the concentrations of 100 and 250 ppm. The PEE concentration of 500 ppm., reduced the bacterial growth more than the concentrations of 100, 250 ppm., and antibiotics after 72 hours, while the PEE concentrations of 750 and 1000 ppm., prevented the bacterial growth in the inhibition zones areas for the treated plates. It means that the high

concentrations of propolis (Egyptian or Bulgarian) were very effective and prevented the Gram positive bacterial growth.

REFERENCES

Abd-Al-Fattah, M.A., Hemeida, H.H. and Nour, M.E. (1993). The Antimicrobial and antioxidant activity of propolis as a natural honey bee product. Bull. Fac. Agric., Univ. Cairo, 44: 637-648.

Abo-EL-Dahab, M.K. and EL-Goorani, M.A. (1984). The bacteria. Dar EL-Maaref, Cairo, Egypt, P. 97-153.

Abu Fares, R.J., Nazer, I.K., Darwish, R.M. and Abu Zarqa, M. (2008). Honey bee hive modification for propolis collection. Jordan Journal of Agricultural Sciences, 4(2): 138-147.

Alppi, A.M. (1991). A comparison of laboratory techniques for the detection of significant bacteria of honey bee, *Apis mellifera* in Argentina. J. Apic. Res., 30 (2): 75-80.

Ashour, A.T.(1989). Studies on propolis gathering with reference to its antimicrobial properties. M.Sc. Thesis, Faculty of Agric., Cairo Univ.

Azimi, PH., Koranyi, K. and Lindsey, K. D. (2008). *Listeria monocytogens*: Synergistic Effects of Ampicillin and Gentamicin. Am. J. Clin. Pathol., 72(6): 974 -977.

Bailey, L. and Lee, D.C. (1962). *Bacillus larvae*: Its cultivation in vitro and its growth in vivo. Journal of General microbiology, 29:711-717.

Bosio, K., Avanzini, C., Avolio, A., Ozino, O. and Savoia, D. (2000). In vitro activity of propolis against *Streptococcus pyogenes*. Appilied Microbiology, 31(2): 174-177.

Cox, B., Eischen, F. and Graham, H. (2005). American Foulbrood Survey in Honeybee Pollinating California Almonds. American Bee Journal, 145 (6): 487 – 488.

Dawoud, E.I., El-Gizawy, S.A and Nour, M.E. (1995). Antimicrobial activity of propolis extract. J. Agric. Sci. Mansoura Univ., 20(5): 2313-2320.

Haynes, W.C. (1972). The catalase test: an aid in the identification of *Bacillus larvae*. American Bee Journal, 112:130-131.

Holst, E.C. (1646). A single field test for American foulbrood. American Bee Journal, 86: 14, 34.

Jelinski, M. (2003). Survival of *Paenibacillus larvae* Sub sp. Larvae Endospores in Honey Substitute Obtained from Bee Colonies Affected with American Foulbrood, Bull. Vet. Inst. Pulawy, 47: 271-273.

Morse, R.A. and Nowogrodzki, R. (1990). Honey bee pests, predators and diseases, second edition. Cornell Univ., press Ithaca and London, p. 29-47.

Owayss, A.A. (2007). Prelimenary investigation on American Foulbrood disease: Recording of the Infection in the Apiaries of Fayoum Governorate. Annals of Agric. Sc., Moshtohor, 45 (2): 903 – 910.

Peters, M., Kilwinski, J., Beringhff, 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 distrtrict of Arnsberg (North Rhine-Westphalia). J. Vet. Med. B 53: 100-104.

Salah, A.G., Abdul-Raouf, U.M., Hassan, R. El-S. and Bakhiet, S.K. (2007). In Vitro Antibacterial Activities of rifampicin and propolis on MRSA (methicillin resistant *Staphylococcus aureus*). Egypt. J. of Appl. Sci., 22(68): 774 -786.

Serra, B. J., Ventura, C. F. and Escola, J.R. (1994). The composition, active components and bacteriostatic activity of Propolis in dietetics. Journal of the American Oil Chemists Society, 71 (5): 529 – 532

Shimanuki, H. and Konx, D.A. (1994). Susceptibility of *Bacillus larva* to Terramycin. American Bee Journal, 134:125-126.

Shimanuki, H. and Konx, D.A. (2000). Diagnosis of honey bee diseases. U.S. department of Agriculture, Agriculture Handbook No. AH- 690, 61 pp.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (1986). Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, MD 21202, USA., 2, P. 1104-1132.

Traynor, K.S. (2008). American foulbrood: How to discover AFB before clinical symptoms. American Bee Journal, 148(11): 1013-1017.

Zakaria, M.E. (2007). The cellular immunity responses in the haemolymph of honey bee workers infected by American foulbrood disease (AFB). Journal of Applied Sciences Research, 3(1): 56-63.

التعرف على بكتيريا *paenibacillus larvae* كمسبب لمرض تعفن الحضنه الامريكي في طوائف نحل العسل ودراسة نشاط البروبوليس المضاد للبكتيريا

شيماء ناجى مصطفى* – اسامه الانصارى* – احمد عبد الحليم الشيمى و محمد عطيه عويس****

*** قسم الحشرات الاقتصاديه – كلية الزراعه – جامعة الاسكندريه – مصر**

****قسم الحشرات الاقتصاديه و المبيدات – كلية الزراعه – جامعة القاهره – جيزه - مصر**

في هذا البحث تم عزل والتعرف على البكتيريا المسببه لمرض تعفن الحضنة الامريكي في طوائف نحل العسل وقد تم التعرف على بكتيريا *paenibacillus larvae* في طوائف نحل العسل في محافظة الأسكندرية وذلك عن طريق اجراء اختبارات عديده وهي: إختبار روبي ، إختبار هولست ، العزل علي بيئات إختيارية ، إنتاج الكتاليز، التحلل المائي للنشا والفحص الميكروسكوبي والذي يتضمن طريقة الصبغ السالب ، صبغ الجراثيم و إختبار الصبغ لجرام.

وكذلك تم اجراء مقارنة بين البروبوليس المصري والبروبوليس المستورد من بلغاريا وكذلك ثلاثة مضادات حيويه وهي التيلوزين والبنسيلين ومخلوط الامبسيلين والجنتاميسين من حيث تأثيرها على ثلاثة انواع من البكتيريا هي *paenibacillus larvae* و *Staphylococcus aureus* و *Listeria monocytogenes* وذلك عن طريق تقنيه قياس قطر منطقة التثبيط للنمو البكتيري . وقد اوضحت النتائج ان مستخلص البروبوليس البلغاري كان اكثر فاعلية من مستخلص البروبوليس المصري. كما أن بكتيريا *S. aureus* كانت أكثر حساسيه لمستخلص البروبوليس الإيثانولي عن *L. monocytogenes* في حين أن *P. larvae* كانت أكثر مقاومة لمستخلص البروبوليس الإيثانولي.