Screening the Efficacy of Different Anti-biotics Against American Foulbrood in Jordan

دراسة مسحية لفاعلية مضادات حيوية مختلفة لمعالجة عفن الحضنة الأمريكي في الأردن

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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 disease is caused by a spore forming bacterium *Peanibacillus larvae* that affects honeybee larvae. This work includes the screening of several alternative antibiotics efficacy to AFB disease. The sensitivity of *Peanibacillus larvae* to seven antibiotics was investigated using disk diffusion methods (Kirby Bauer disk diffusion). The tested compounds are approved by the US - Food and Drug Administration for agricultures uses. The most active antibiotics were Novobiocin and Florfenicol followed by Oxytetracycline and Tylosin. Erythromycin and Tilmicosin had intermediate activity; Lincomycin was recorded as the lowest active

antibiotic against *P.larvae*. The result showed that Oxytetracycline resistance is still not a problem in Jordan, unlike in the USA and some other regions. To avoid any further problems in the spread of antibiotics-resistance, it is recommended that beekeepers have to establish methods for early detection of AFB, the application of antibiotics in inadequate doses with technical supervision and using of different alternative antibiotics such as Tylosin. Further studies are needed to determine the fate of Oxytetracycline and Tylosin residues in honey under field conditions at the recommended doses for field trial; however we do not recommend the prophylactic use of antibiotics whereas we recommend the treatment only in diseased colonies with high honey bee population.

Keywords: American Foulbrood Disease, Antibiotics-Resistance, Honey Bee, Jordan.

ملخص

يعتبر عفن الحضنة الأمريكي واحد من أهم الممرضات البكتيرية التي تصيب نحل العسل مسببة خسائر كبرى لقطاع تربية النحل على الصعيد العالمي. وتعتبر سبورات بكتيريا (*Peanibacillus larvae*) السبب الرئيس وراء إصابة يرقات النحل بهذا المرض. في هذه الدراسة تم مسح تأثير سبعة المضادات الحيوية بإستخدام طريقة قرص الإنتشار (Kirby Bauer disk diffusion). وتعتبر المضادات المستخدمة هي تلك المعتمدة من خلال مؤسسة الغذاء والدواء الأمريكية ومعتمدة للاستعمال في القطاع الزراعي. وقد تبين أن مضادات (Novobiocin) و(Florfenicol) متبوعة بـ (Oxytetracycline) ثم (Tylosin). في حين كان كل من (Erythromycin) و(Tilmicosin) محدودة الفاعلية، وكان المضاد الحيوي (Lincomycin) الأقل فاعلية. وبذلك تبين أن (Oxytetracycline) لا يزال فاعل ولم تتشكل لدى البكتيريا مناعة ضدة بعكس الحالة الحاصلة في الولايات المتحدة الأمريكية وبعض الأقاليم الأخرى.

لمنع تكون مناعة عند البكتيريا ضد المضاد الحيوي (Oxytetracycline) الاكثر شيوعاً في الاستخدام بين مربي النحل في الأردن وبلاد الشام، لا بد من إرشاد مربي النحل إلى المستويات الدقيقة لاستخدام هذا المضاد ومداورة استخدامه بمضاد ال (Tylosin). لابد من إجراء دراسات ميدانية تتبع هذه المخبرية لتحديد مستويات تلوث منتجات النحل بهذه المضادات ولتحديد النسب اوالطرق الأنجع في الإستخدام، وتبقى التوصية الأساس عدم إستخدام المضادرات الحيوية بأهداف وقائية وإمكانية إستخدامها لاغراض علاجية في الخلايا المصابة والتي تتمتع بكثافة نحل عالية.

Introduction

Honey bees are playing a vital role in the agricultural sector by pollinating crops and fruit trees (Shammout, *et al.* 2014), 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 is an infectious disease of the larval stage of the honeybee *A. mellifera,* and occurs throughout the world where such bees are kept (OIE, 2008)**.** There is no seasonal outbreak of AFB; it occurs at any time of the year when brood is present (Bailey, & Ball, 1991), but it is usually diagnosed during the active brood-rearing season (Adjlane, *et al*. 2012). It is well established that young honeybee larvae are highly susceptible while older larvae and adult bees, even adults that transfer the spores and have close contact with young larvae, never become infected (Brodsgaard, *et al.* 2000). AFB that is caused by the gram-positive bacterium *Paenibacillus larvae* has a significant capacity to spread. It can produce billions of spores in each infected larva, and can survive for many years in scales, hive products and equipment used by beekeepers. Moreover, this bacterium is very resistance to heat and chemicals (Brodsgaard & Hansen, 2003).

Antibiotics are routinely used to control AFB in honeybees, but rapid development of antibiotic-resistant microbial strains and growing concern for residues in honeybee products promoted the need to investigate the use of alternate antibiotics.

In order to minimize financial loss of beekeepers, antimicrobial and antibiotic therapy for prevention and treatment of AFB has been

investigated since 1940 (Kochansky, 2001). Several types of antibiotics were and are still used to control AFB disease; *P.larvae* was screened for sensitivity to antibiotics based on the size of inhibition zone on agars plate.

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. Beekeepers in Jordan generally use antibiotics to control (AFB) disease. Recently, increasing incidences of (AFB) and problems controlling the disease – based on resistance build-up in bacteria, increased residue problems, and loss of natural resistance in bees – have renewed the interest for AFB tolerant honeybee worldwide. According to a "Strengths, Weaknesses, Opportunities and Threats analysis" (SWAT) carried out by the bee research unit of National Center for Agriculture Research and Extension (NCARE) during the winter of 2008-2009, beekeepers do have a severe problem with the treatment of the AFB, underlining the need to carry out further research (Haddad, N. 2015). The main antibiotic used in Jordan is Oxtetracycline (OTC). Oxytetracyclin is a broad-spectrum antibiotic that is active against a wide variety of bacteria; however, some strains of bacteria have developed resistance to this antibiotic, which has reduced its effectiveness for treating some types of infection. The conventional antibiotic, Oxtetracycline remains the only registered antibiotic to control AFB in honeybees in the USA also, but the development of the OTC resistant bacterial strains lessens its efficacy. Resistance to OTC has been reported (Miyagi, *et al.* 1999) and tests by the Bee Disease Diagnosis Service of the USDA Bee Research Laboratory have shown it to be widespread in the USA and Canada. Because of OTC resistance, and in order to have alternative antibiotics available, screens of various antibiotics will be carried out in this study. According to Jordanian beekeepers and after interviewing them about the efficiency of Oxtetracycline (OTC) as a treatment to AFB, the majority of them declared that it is of little use.

The objectives of this project is:

- Evaluate the resistance of AFB in Jordan to different antibiotics, including the effect of oxytetracycline on the *Paenibacillus* larvae.
- Determine the Minimum Inhibitory Concentration for some antibiotics.
- Eventually, recommendations will be provided to beekeepers about the most appropriate antibiotics and their doses.

Materials and methods

Isolation of *P. larvae*

Larvae with clinical symptoms of AFB were removed by using a toothpick and then mixed well with 1 ml of sterile distilled water (SDW) (2 larval remains per tube). 100 µl of the suspension was diluted in 900 SDW, vortex mixed, then the suspension were centrifuged 2000 rpm for 5 min. (Allipi, 2002). 200µl from each sample were inoculated over the surface of the MYPGP media (Mueller-Hinton, Yeast extract, phosphate of potassium, Glucose and Pyruvate, and then incubated at (34-37) °C for 2-4 days (CO2 incubator, USA). Colonies morphology appeared small, regular, mostly rough, flat or raised and whitish to beige colored.

Disk Antibiogram of *P.larvae* isolates

Seven antibiotics approved by the United States Food and Drug Administration (FDA) for agriculture uses (Kochansky, *et al.* 2005), were tested against *P.larvae* by Disk Diffusion method. AFB spores suspensions were prepared by inoculated *P.larvae* (freshly media) in 5ml Muller Hinton broth and adjusted to the turbidity of Mcfarland 0.5 standard (0.05 ml barium chloride (1%) and 9.95 sulfuric acid (1%)). This turbidity was equivalent to 108 CFU (colony forming Units)/ml, 200µl of the suspension was spread over the surface of freshly prepared Brain heart- infusion agar (BHIT) plates, with *P.larvae* by sterile cotton swab. Using sterile forceps and under aseptic conditions. Antibiotics (Table 1) disks were placed in duplicates on the surface of each inoculated agar plate, and were pressed gently with the tip of the forceps

to ensure intimate contact. Finally, the plate incubated at 34º C in the dark. The diameters of zone inhibition were measured after 24h (Kochansky, *et al.* 2005). Antibiotics used in disk diffusion method and their concentration were presented in table 2.

Table (1): Classes of antibiotics tested vs. American foulbrood.

Classes	The antibiotics used
Chloramphenicol analogs	Florfenicol
Lincosaminisdes	Lincomycin
Macrolides	Tylosin
Tetracycline	Oxytetracycline
Miscellaneous	Novobiocin Erythromcosin Tilmicosin

Table (2): Antibiotics used in disk diffusion method and their concentration.

Antibiotics	Concentration	Abbreviation
Erythromycin	15 MCG	E
Oxytetracycline	30 MCG	OT
Tylosin	30 MCG	TY
Lincomycin	2 MCG	LN
Florfenical	30 MCG	FFC
Tilmicosin	2 MCG	TEF
novobiocin	5 MCG	NV

Minimum inhibitory concentration (MIC)

According to two several previous studies, Oxytetrcyclin (OTC), and Tylosin (TY) were tested to determinate Minimum inhibitory concentration (MIC). Muller Hinton broth (5ml) was inoculated with *P.larvae* and adjusted to the turbidity of Mcfarland 0.5 turbidity standard (as described in the previous step). This turbidity was equivalent to 108 CFU/ml. cultures were then used for inoculation of plates of Muller Hinton agar in duplicates, which contained different concentration of

Oxytetracyclin and Tylosin, standard loop that delivers 0.001 ml of culture were used.

Inoculated plates were incubated at 34 C for 24 hr. MIC was read as the lowest concentration of both antibiotics at which complete inhibition of growth occurs on the plate. Prepared media containing 5µg of OTC (Kochansky, *et al*. 2005) and media without any concentration of antibiotics were used as positive controls (with no growth appearance).

Results

Disk Diffusion Method

The sensitivity of *Peanibacillus larvae* positive isolates (25 samples) were tested by disk diffusion method to seven US FDA administration approved antibiotics. The seven antibiotic disks gave a visible zone of inhibition around the disk, the total diameter of that zone was recorded and the compound was termed as "active" at the antibiotic concentrations, this result included that there were several alternative antibiotics that may be used as a treatment for AFB. The most active antibiotics that completely inhibited the growth of *P.larvae* were Novobiocin and Florfenicol (FFC) (100%) followed by Oxytetracycline (OTC) and Tylosin (TY) (98%). Isolated *P.larvae* did not show any resistance to the antibiotics OTC. The lowest active antibiotics were Lincomycin (LN) (4%). Antibiotics remains gave 84% and 80% for Tilmicosin (TEL), Erythromycin (E) respectively (Table 3-4, Fig. 1).

Table (3). Antimicrobial susceptibility of *Peanibacillus larvae* to the seven approved antibiotics and their inhibition zone diameter (mm).

Sample No	OTC	TY	E	TEL	FFC	LN	NV
1BN	15	14	23	13	26	5	19
2B	20	28	21	23	35	23	16
1L	29	22	20	18	26	6	17
10 BN	19	24	17	18	30	11	19
15 BN	26	24	22	20	24	0	16
12 BN	27	25	22	12	25	10	18

…continue table (1)

Sample No	OTC	TY	E	TEL	FFC	LN	NV
16 BN	30	33	23	22	29	13	17
19 BN	30	22	22	17	27	10	16
20 BN	29	22	19	17	28	0	19
21 BN	32	22	16	17	28	5	16
22 BN	23	19	22	20	29	6	19
24 BN	29	23	19	18	31	10	16
27 BN	29	26	22	17	28	9	17
32 BN	24	22	19	17	29	11	20
34 BN	18	21	22	14	23	5	18
36 BN	25	27	22	21	25	5	18
37 BN	28	24	18	18	29	7	16
38 BN	29	24	18	13	27	13	18
40 BN	28	25	20	20	25	10	18
42 BN	27	21	21	22	26	9	20
43 BN	32	25	26	24	28	5	17
45 BN	25	25	21	12	26	13	17
46 BN	28	32	16	16	26	13	17
3 L	29	23	14	14	26	10	18
4 L	28	22	17	12	32	9	20

BN: brood-nest honey samples;

B: bulk honey samples;

L: larval remains.

Table (4): Inhibition zone diameter of different antibiotics against *Peanibacillus larvae.*

Inhibition zone diameter Range	**Antibiotics and number of isolates in each range**						
	OTC	TY	E	TEL	FFC	LN	NV
0-5 mm	_	_	_	_	_	7	_
6- 15 mm	1	1	1	4	_	17	_
16-25 mm	7	20	23	20	5	1	25
16-35 mm	17	4	1	1	20	_	_

(Disk Diffusion Methods);
OTC : Oxytetracycline ;
TY: Tylosin ;
E : Erythromycin ;
TEL : Tilmicosin ;
FFC : Florfenicol ;
LN : Lincomycin ;
NV : Novobiocin.

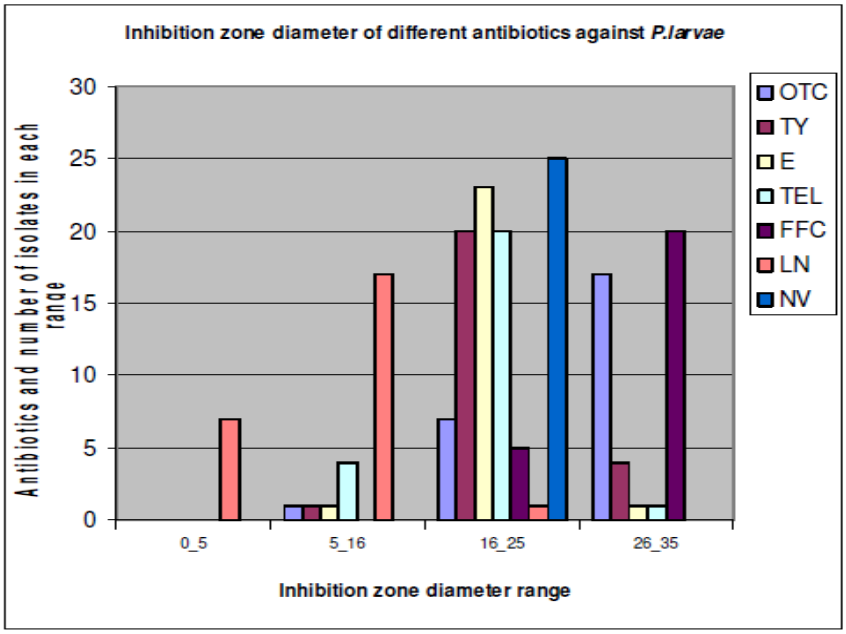

Figure (1): Inhibition zone diameter of different antibiotics against *Peanibacillus larvae.*

(Disk Diffusion Methods);
OTC : Oxytetracycline ;
TY: Tylosin ; E :
Erythromycin ; TEL :
Tilmicosin ;
FFC : Florfenicol ;
LN : Lincomycin ;
NV : Novobiocin.

According to table 4, all antibiotics worked in the inhibition zone diameter range (16-25).

The percentage of sensitivity equalizes to number of samples with inhibition zone higher than 16 mm division by total sample number (25) multiplied with 100% (Table 5).

Table (5) The zone diameter for each antibiotic with their susceptible (sensitive) percentage.

Antibiotics	Zone diameter			Percentage of sensitive isolates
	Susceptible	Intermediate	Resistance	
OTC	> 19	15-18	< 16	92 %
TY	> 20	15-18	< 16	92 %
E	> 18	14-17	< 13	80 %
TEL	> 14	11-13	< 10	84 %
FFC	> 19	*		100 %
LN	> 15	10-14	< 9	4 %
NV	> 16	15-16	< 14	100 %
Ranges given clinical and laboratory institute (CLSI), performance standards for antimicrobial disk susceptibility testing M2 –A8 , 2003 and zone of inhibitions for various antibiotics – Kirby bauer disk diffusion • Non value were found				

OTC : Oxytetracycline ;
TY: Tylosin ;
E : Erythromycin ;
TEL : Tilmicosin ;
FFC : Florfenicol ;
LN : Lincomycin ;
NV : Novobiocin.

2.2. Determination of Minimum Inhibitory Concentration (MIC)

Peanibacillus larvae were treated with different concentration of Oxytetracycline and tylosin to determine the MIC with the range of (8-0.04) μg/ml. The result showed that both antibiotics were active in concentrations as low as 0.04 μg/ml.(Table 6).

Table (6): Comparison of activity of OTC & TY antibiotics against *Peanibacillus larvae* Samples.

	Antibiotics dose (µg/ml)								
	8	4	1.2	0.4	0.12	0.04	0.012	0.004	N
Oxytetracycline (OTC)	Active	Active	Active	Active	Active	Active	Inactive	Inactive	2
Tylosin (TY)	Active	Active	Active	Active	Active	Active	Inactive	Inactive	2
N: number of replicates for each antibiotics									

Discussion

While different culture methods have been proposed for controlling AFB including the breeding of hygienic bees that diligently remove AFB- infected larva (Spivak & Reuter, 2001), burning of infected colonies (that is costly for beekeepers) and shaking methods, antibiotics still remain a valuable tool in the control of AFB (Genersch, 2010).

However, treatment with antibiotics have some limits, which include the appearance of antibiotics strains resistance which will inhibit the bacterial response to the antibiotics that may be related to the misused of antibiotics in an overdose or in a wrong application of it to the bee colonies.

In Jordan two main antibiotics are currently being used in the treatment of AFB; Oxytetracycline and sulfathiazole. OTC resistance strains of the bacteria were reported by Alippi (2000). Sulfathiazole lost the approval by FAD 30 years ago (Kochansky, *et al*. 2005). This study was carried out to investigate the presence of Oxytetracycline resistance in *P.larvae* isolated from honey samples in Jordan and to screening of alternative antibiotics against AFB.

Development of resistance strains of *P.larvae* has been reported in many countries, the first investigation was in Argentina (Alippi, 2000), and the researcher suggest that other antibiotics should be investigated for future control of AFB. In a study of Kochansky, *et al*. (2005), 35 FAD approval antibiotics and synthetic antimicrobials were investigated against *P.larvae* by disk diffusion methods to screened alternative

antibiotics to Oxytetracycline (OTC). The most active approved antibiotics screened were Pirlimycin and Tiamulin (0.12 µg /disk) that showed high activity and shared the mode of action of Tylosin (0.04 µg /disk) and Lincomycin by inhibition of ribosomal peptides synthesis, so both of them offers no advantage over Tylosin.

The results of the seven US FAD (United States Food and Drug Administration) approved antibiotics that were investigated by disk diffusion methods against *P.larvae* isolates from different region of Jordan showed different actions of antibiotics regarding to antibiotics mode actions on bacteria. Since Oxytetracycline (OTC) is often used to prevent AFB, in this study susceptibility to OTC was assessed by the disk diffusion methods of *P.*larvae positive isolates. All isolates were susceptible to 30 µg of OTC, and MIC was < 0.04 µg /ml, Table (4, 5). The results indicate that OTC resistance is still not a problem in Jordanian regions.

According to a survey which made in Jordan about honeybees disease, not all Jordanian beekeepers used OTC to deal with AFB problems. Three Macrocyclic lactones were studied; Tylosin, The result show that TY had similar effects with OTC on isolated samples with susceptibility to 30 µg of TY and MIC < 0.04 µg/ml (Table 4, 5). These values indicated that very low concentration of tylosin is required to inhibit the growth of *P.larvae*. Tylosin has been shown to be relatively non-toxic to bees and effective in controlling AFB and less toxic to honeybee larvae than Oxytetracycline (Hitchcock, *et al.* 1970), thus Tylosin is potentially a suitable alternative to Oxytetracycline with the same activity but less toxicity. Alippi, *et al*. (2005) showed that Tylosin treatment could be effective in vivo and there is no negative effects in colonies were noted to any dosage rates or forms of application, and the MIC values ranging from 0.0078 and 0.5 µg/ml depending upon the tested strains.

In 2004 Kochansky studied another macrolides Desmycosin that gave equal activity to Tylosin (down to 0.04 µg/disk), Desmycosin was not subjected to field trails because it is not commercially available and has no FDA approvals. Thus result showed that using of Tylosin will be

effective against AFB instead of OTC. Also the antibiotic Tylosin was approved to use as alternative antibiotic to OTC for the control of AFB in several studies (Elzen, *et al.* 2002; Alippi, *et al.* 2005).

Tilmicosin is a smi-synthatic macrolide synthesized by a chemical modification of Tylosin related compound. Fransico, *et al*. 2008, assessed the response of 23 strains of *P.*larvae from; diverse geographical origins to tilmicosin that considered being non-toxic for adults bee and also for larvae, all 23 strains tested showed all susceptibility to tilmicosin with inhibition zone diameter between 21 and 50 mm. In this study tilmicosin showed 84% Susceptibility of *P.larvae* with inhibition zone diameter from 26 to 35, the differences of the diameter may relate to the strains difference. Laboratory screening of antibiotics indicated that the lincosaminide lincomycin effectively inhibited the growth of OTC (Kochansky, 2001). Feldlaufer, *et al.* also in 2001, was tried to evaluate the antibiotics lincomycin for the toxicity to larval and adult honey bees and for efficacy in controlling American foul brood (AFB) in USA, the result included that the treatment with lincomycin had no toxicity on both larvae and adult honey bees or their mortality, and when it was used as a treatment to AFB (antibiotics with sugars) no visible signs of AFB could be found after 54 days from treatment. In this study the lincomycin showed low percent with (4%) with a low range 5-15 mm, that indicate that the existence of different strains comparing with USA.

The use of florfenicol in this study was to compare the result with previous research, which give the highest susceptibly percent Florfenicol 30 µg/disk (100%) with rang 16-35 mm for all *P.larvae* isolates. In 2005, Kochansky showed that florfenicol was active down to 1.2 µg/disk that it was considered as poor activity. Despite the high activity of florfenicol on AFB, its residue in honey will be one of the most important prevention to use it.

The Miscellaneous Novobiocin 5 µg/disk with the range of 16-25 gave 100% susceptibly to *P.larvae* isolates. Kochansky in 2004 had the same result which include that Novobiocin was only active at the highest dose of 4 µg/disk. Using of high dose cause residues problem, for that Novobiocin its offer no advantage to use instead of OTC.

Finally, *Peanibacillus larvae* OTC resistance is still not a problem in Jordan, OTC susceptible care must be taken to avoid the application of the antibiotics of poor quality, in inadequate doses, and without technical supervision. Using of alternative antibiotics such as Tylosin will help to avoid resistance formation by the bacterium in the recent years. Further studies on OTC and TY residues in honey need to be conducted.

Conclusion

Oxytetracycline is the main antibiotics used in Jordan against AFB, and no resistance strains were found during the study period. The most active antibiotics were Novobiocin and florfenicol followed by Oxytetracycline and Tylosin. Erythromycin and Tilmicosin have intermediate activity; Lincomycin was recorded as the lowest effective antibiotic against AFB. We highly recommend to beekeepers to practice rotate in the usage of Tylosin and oxytetracycline to prevent future resistance to one of these antibiotics. It is also crucial for beekeepers to avoid prophylactic use of antibiotics in healthy colonies to avoid resistance to antibiotics and also to prevent any honey contamination with antibiotics. Further studies are needed to determine the fate of the used antibiotics in honey under field Conditions, whoever we do not recommend the prophylactic use of antibiotics whereas we recommend the treatment only in diseased colonies with high honey bee population.

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Competing Interests

The authors have declared that no competing interest exists.

References

- Adjlane, N. kechih, S. Doumandji, S.E. & Haddad, N. (2012). Survey of American foulbrood in colonies in mid-northern region of Algeria. *Uludag Bee Journal. 12(3)*: 98-105 98.
- Alippi, A.M. (2000). Is Terramicyn1 losing its effectiveness against AFB? The Argentinian experience. *Bee Biz 11*. 27–29.
- Alippi, A. M. (1995). Detection of bacillus larvae spores in Argentinain honeys by using a *semiselective* medium, *Microbiologia 11(3)*. 14-18.
- Alippi, A. M. & Reynaldi, F. J. (2005). Inhibition of the growth of Paenibacillus larvae, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore forming Bacteria isolated from apiarian sources. *Journal of Invertebrate Pathology 91*. 123-126.
- Arabiat, A-H. (2007). *Prevalence and Diagnosis of Foulbrood Diseases in Jordanian Honey Bees, Based on Polymerase Chain Reaction (PCR) Technique*. PhD. Thesis Al Balqa University. Salt - Jordan.
- Bailey, L. & Ball, B.V. (1991). *Honey Bee Pathology*. Academic Press. London - New York, 125 p.
- Broodsgard, C.J. Hanen, H. & Ritter, W. (2000). Progress of Paenibacillus larvae larvae infection in individually inoculated honey bee larvae reared single in vitro, in micro colonies, or in full-size colonies. *Journal of Apicultural Research. 39*. 19 - 27.
- Brodsgaard, C. & Hansen, H. (2003) *Tolerance mechanisms against merican Foulbrood in Honey bee larvae and colonies. Apiacta, 38*. 12-19.
- Elzen, P. J. Westervelt, D. Causey, J. Hepburn, H. R. & Neumann, P. (2002). Method of Application of Tylosin, an Antibiotic for American Foulbrood Control, with Effects on Small Hive Beetle

(*Coleoptera: Nitidulidae*) Populations. *Apicultural and Social Insects, 12*. 34-54.

- Mark, F. Feldlaufer, Jeffery S. Pettis, J. Kochansky, P. & Grant, S. (2001). Lincomycin hydrochloride for the control of American foulbrood disease of honey bees. *Apidologie: 32 (6)*. 457-554.
- Reynaldi, F.J. Graciela, N. Albo, G.N. & Alippi, M.A. (2008). *Effectiveness of tilmicosin against Paenibacillus larvae, the causal agent of American Foulbrood disease of honeybees*. *Veterinary Microbiology*, 15. 89-102.
- Genersch. E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrale Pathology, 103*. 10–19.
- Haddad. N. Brake. M. Megrade. H. & De Miranda. J. (2008). The First Detection of Honeybee Viral Diseases in Jordan using the PCR. *Jordan Journal Agriculture and Science, 4*. 57 – 61.
- Hitchocok, J.D. Moffet, J.O. Lackett, J.J. & Elliot, J.R. (1970). Tylosin fdor control of Americaan foulbrood disease in honey bees. *Journal of Economy Entomolology, 63*. 204 – 207.
- Kochansky, Pettis J. (2005). Screening additional antibiotics for efficacy against American foulbrood. *Journal of Apicultural Research, 34*. 89-112.
- Kochansky, J. Knox, D.A. Feldlaufer, M. & Pettis J. (2010). Screening alternative antibiotics against oxytetracycline-susceptible and -resistant *Paenibacillus larvae*. *Apidologie, 12*. 89-99.
- Kochansky, J. (2004). Degradation of tylosin residues in honey. *Journal of Apicultural Research, 24*. 123-129.
- Miyagi, T. Peng, C.Y.S. Chuang, R.Y. Mussen, E.C. Spivak, M.S. & Doi, R.H. (1999). Verification of Oxytetracycline-resistant American Foulbrood Pathogen Paenibacillus larvae in the United States. *Journal of Invertebrate. Pathology, 75*. 95 - 96.

- Office International des Epizooties (OIE). (2008). *American foulbrood of honeybees.* Chapter 2.2.2 in Manual of diagnostic tests and vaccines for terrestrial animals.
- Otten, C. (2003). A general overview on AFB and EFB pathogen, way of infection, multiplication, Clinical symptoms and outbreak. *Journal of Apicultural Research, 54(9).* 234-239.
- Spivak, M.S. & Reuter, G.S. (2001). Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie, 32*. 555 – 565.
- Shammout, A. Haddad, N. & Abuobeid, O. (2014). The Monetary Value of Ecosystem Services Provided by Insects, "A case study for selected crops in Jordan. *Jordan Journal of Agricultural Sciences. 10*(1). 16-33.
- Waite, R. Brown, M. Thompson, H. & Bew, M. (2003). Control of American foulbrood by eradication of infected colonies. *Apiacta, 38*. 134-136.