

# Isolation and identification of *Melissococcus plutonius* from European foulbrood infected beehives and in vivo using of oxytetracycline for treatment

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## Abstract

European foulbrood (EFB) is an infectious and contagious bacterial disease of honeybee larvae caused by (*Melissococcus plutonius*) affects primarily the young larvae (less than 48 hrs. old), and kill mainly the unsealed larvae at the age of 4 to 5 days. The aim of the present study was the isolation and diagnosis of *M. plutonius* that cause European foulbrood disease in beehives and used oxytetracycline in treatment of this disease (*in vivo*). The infected larvae were detected by the clinical signs appears; firstly the larvae become flaccid and twisted in C-shape, the affected brood appeared yellowish, cream-colored, and later brown to almost black that can easily be removed from the cells. Sixty nine swabs samples were collected from bees cells in Hila city. Samples were cultured on selective, differential media, and biochemical tests. All positive samples for *M. plutonius*, were subcultures on nutrient agar, blood agar and yeast extract agar, and incubated at 35°C for a week under anaerobic condition. Results declare presence of 57 samples (82.6%) give positive result of *M. plutonius*, while 12 (17.4%) samples were negative. Bacteria appears as a Gram-positive lanceolatecoccus, slender rods with a tendency to form chains. Results also showed that nine days treatment with oxytertracycline decrease the mortality rate, the disease was disappear, and the infected larvae sealed and returned to normal. In conclusion: The study found that the EFB was decreased when oxytetracycline used as treatment.

**Key words:** European foulbrood disease, oxytetracycline, *M. plutonius*, larvae, beehives.

## عزل وتشخيص *Melissococcus plutonius* من مرض تعفن الحضنة الاوربي في خلايا النحل وتأثير استخدام الاوكسي تتراسايكلين في علاجها (في الجسم الحي)

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## الخلاصة

مرض تعفن الحضنة الاوربي هو مرض جرثومي معدي ليرقات خلايا النحل يصيب اليرقات البدائية (اقل من 48 ساعة) ، ويصيب بصورة رئيسية اليرقات المفتوحة ويقتلها بعمر 4 الى 5 ايام . هدفت الدراسة الى عزل وتشخيص بكتيريا *M. plutonius* من اصابات تعفن الحضنة الاوربي في خلايا النحل واستخدام الاوكسي تتراسايكلين لعلاج المرض (في الجسم الحي). تم تشخيص اليرقة المصابة والكشف عنها من خلال العلامات السريرية التي ظهرت ، حيث تكون اليرقة المصابة في البداية مترهلة و ملتوية على شكل حرف C ولونها أصفر الى كريمي يتحول فيما بعد الى بني ثم الى اسود ويسهل ازالتها من الخلية. جمعت تسع وستون عينة من خلايا نحل في مدينة الحلة. وزرعت هذه العينات على الاوساط التفريقية والانتخابية وأجريت لها الاختبارات الكيموحيوية ، وكل العينات موجبة *M. plutonius* ، زرعت على المرق المغذي ، واكار الدم ، ومستخلص اكار الخميرة ، وحضنت بدرجة 35° درجة مئوية لمدة اسبوع في الظروف اللاهوائية. اظهرت النتائج وجود 57 (82.6%) عينة موجبة لبكتيريا *M. plutonius* ، بينما 12 (17.4%) عينة كانت سالبة. وكانت البكتريا موجبة لصبغة كرام وكروية واسطوانية عصوية الشكل حيث تميل الى شكل السلاسل. كما اظهرت النتائج ان (9) ايام من العلاج بالاوكسي تتراسايكلين ادى الى خفض نسبة الهلاكات ، وغلق اليرقات وعودتها الى حالها الطبيعي ، واختفاء المرض. نستنتج من الدراسة ان مرض تعفن الحضنة الاوربي يقل عندما نستخدم الاوكسي تتراسايكلين في العلاج. الكلمات المفتاحية: مرض تعفن الحضنة الاوربي ، الاوكسي تتراسايكلين ، ملي كوكز بليتونيس ، اليرقة ، خلايا النحل.

## Introduction

Honey bees (*Apis mellifera*) are not only important for the produced honey but they are also vital as pollinators of agricultural and horticultural crops, hence the health of honey bees has a great economic impact worldwide, in recent years, serious losses of bees from beehives and a decline in bee populations have been reported, the cause of these losses is not known, although various hypotheses have been forwarded, large diversity of microorganisms are associated with honey bees (1, 2). Most of them are beneficial but some are also very harmful, honey bee brood and adults are attacked by a range of diseases causing by organisms such as bacteria, viruses, protozoa, fungi and parasitic mites. Two of the most economically important diseases of honey bees are bacterial diseases which affecting the bee brood, American foulbrood (AFB) and European foulbrood (EFB) which are both widely distributed and potentially lethal to infected colonies (3). EFB is an infectious and contagious bacterial disease (*Melissococcus plutonius*) of honeybee larvae, which affects mainly unsealed larvae and kills them at the age of 4 to 5 days, it affecting primarily young larvae (less than 48 hrs. old) but in long established infections, also capped larvae in the latter case. Symptoms are often confused with AFB. However, the causal organism *Melissococcus plutonius*, does not form spores, so the disease is believed to be less problematic and often curable (4). It is the most widespread bacterial brood disease in Great Britain (5), and of specific interest is the development in Switzerland, where the incidence of EFB has constantly risen each year since the late 1990 (6). Although EFB had previously been successfully controlled by sanitizing measures or re queening with a more resistant stock, these methodologies are now proving to be ineffective (7). Oxytetracycline (OTC) has been used to treat EFB for decades, the concentration of OTC in honeybee larvae required to protect them from developing EFB is still unknown. The aims to present up-to-date information on EFB and to synthesize present knowledge of this obscure disease of honey bee, provide an

indicator as to how much OTC is required to protect honeybee larvae from EFB and provide a better guide as to how much OTC is required for hive treatment.

## Materials and methods

### Samples Collection

Sixty nine (69) swabs were collected from infected beehives in Hila city (during a period from February to October 2014). All the infected specimens were young larvae (less than 48 hrs. old). The infected larvae appear flaccid and turn twisted in C- shape. The affected brood appeared yellowish, cream-colored, later become brown to almost black that can easily be removed from the cells. Bacteria was isolated, identified, culturing and biochemically testing using standard procedures. All the samples positive for *M. plutonius* were subcultures on nutrient agar, blood agar and yeast extract agar, and incubated at 35°C for a week under anaerobic condition. All the isolates were stored in brain heart infusion broth with 15% glycerol at -20°C until further use (6).

### Biochemical tests

Conventional methods were used to test for the production of catalase and oxidase. The results of acid production from lactose, D-glucose and D-fructose were further confirmed by conventional methods using carbohydrate test media after two weeks incubation of bacteria at 35°C under anaerobic condition (8).

### Treatment EFB by using oxytetracycline

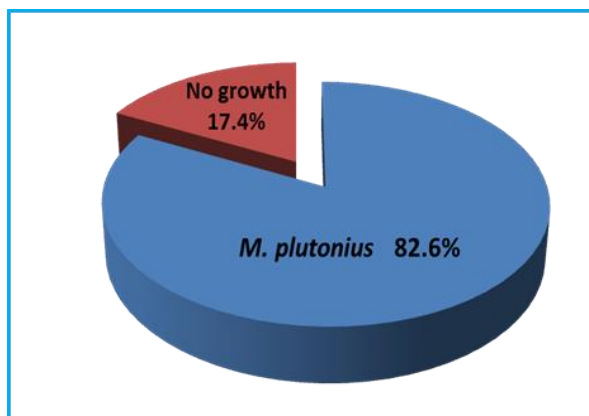
Dry feeding treatment was the only method recommended. Treat each full-sized hive (two-decker) with 1 g of soluble oxytetracycline hydrochloride (OTC) (active ingredient) mixed thoroughly in 100 g of castor sugar. Formulations containing 10g/kg of active OTC made up with castor sugar can be used direct from the container. When mixing antibiotics must be done with caution, using gloves and a face mask. If prolonged transport of home mixed formulations was necessary re-mix the formulation on arrival in case the mixture had been settled out. Apply the dry mixture by sprinkling it between brood nest frames. Queen excluders should be removed before applying the

treatment. Treatments should not be applied by dusting the face of combs with powder. Treated by this way may suffer additional stress when larvae were exposed to concentrated antibiotic. Identify all honey extracted

after any treatments of OTC. The treatment period starts from one day of infected larvae and continues to the ninth day. Ensure when selling bulk honey that this information was made available to the buyer of honey (9).

## Results

Results found that 57 (82.6%) samples were positively infected with *M. plutonius*, while 12(17.4%) gave negative results out of the total (69) samples (Fig. 1).



**Fig. (1): Percentage of bacterial isolates (*M. plutonius*).**

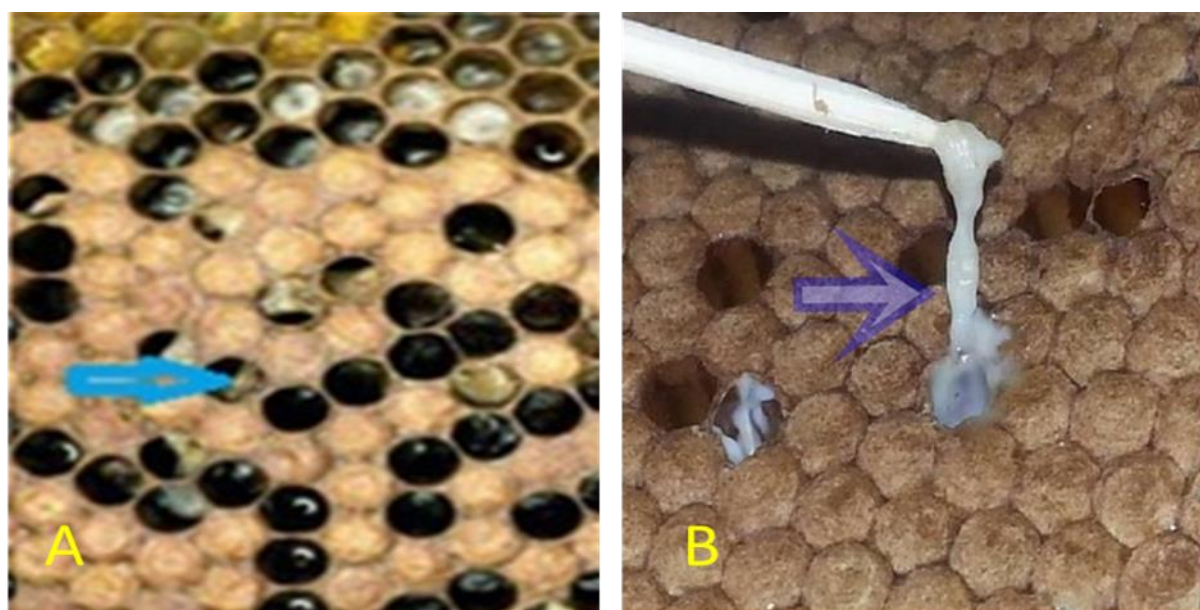
## Symptoms of European foulbrood

Study was declared that the infected larvae which escape detection by adult bees and then die, first become flaccid and turn twisted in C-shape. Affected brood show yellowish, cream-color, later become brown to almost black that can easily be removed

from the cells, slightly ropey with threads less than 1.5cm, decompose, and become granular- watery, tracheal tubes often visible, rubbery and stage of brood before capped, severely affected brood may have a very stale or sour odor, sometimes acidic odor like vinegar due to secondary invaders (Fig. 2).

## Bacterial isolation

The *M. plutonius* pathogen was gram-positive lanceolatecoccus, slender rods with a tendency to form chains, also it was a fastidious organism, requiring microaerophilic to anaerobic conditions and carbon dioxide for growth (Fig. 3). On culture media, individual colonies are small, about 3-4 mm in diameter, opaque, whitish to greyish, flat, with rough surfaces and irregular edge. The laboratory diagnosis was entirely positive after isolation of *M. plutonius* on an artificial nutrient medium. *M. plutonius* is a very exigent microorganism, and could be cultivated on a medium containing yeast extract.



**Fig. (2): Symptoms of European foulbrood. A- Twisted and discolored larvae, B-Slightly ropey with thread less than 1.5 cm.**

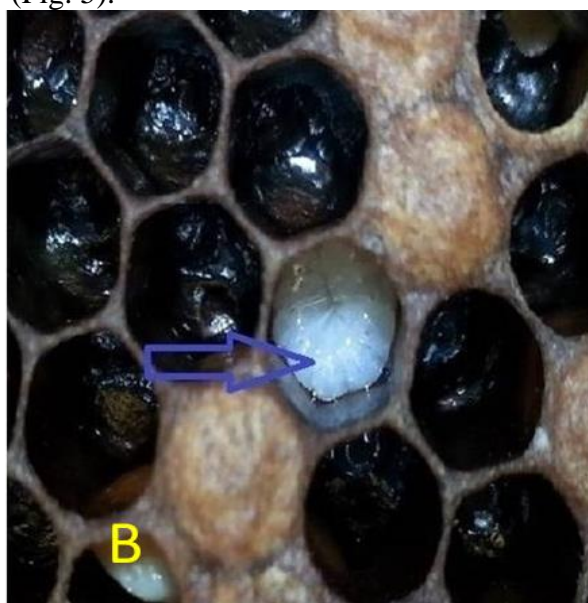
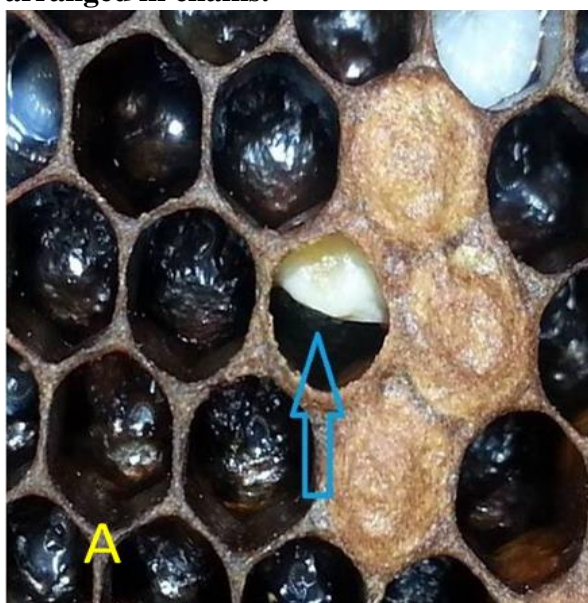




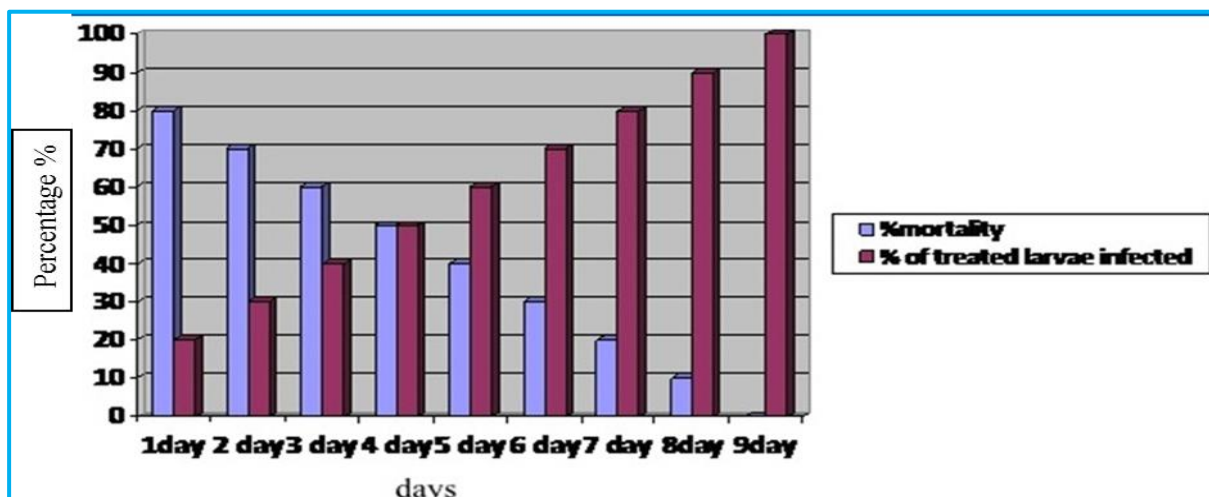
**Fig. (3):** *Melissococcus plutonius* bacteria isolated from infected beehives stained with Gram stain, see the coccus shape arranged in chains.

### Infection and mortality rate of larvae infected with *M. plutonius* and oxytetracycline treatment (OTC)

Before OTC treatment there was elevation in mortality and infection rate of EFB disease, while after treatment the mortality, and infected rate were decrease. The infected larvae appeared unsealed before treatment, and after OTC treatment the larva closed and returned to normal (Fig.4). Results also showed that the infected honeybee larvae with *M. plutonius* need nine days of treatment with OTC to cure and disappear the signs of the disease. Within these nine days the mortality rate decline sharply, contrary to the healing rate which increased sharply from the first to the ninth day of OTC treatment (Fig. 5).



**Fig. (4):** Nature of larvae; A- unsealed larva (before treatment), and B- Sealed larva after nine days of treatment with OTC.



**Fig. (5):** Percentage of mortality and healing rates of honeybee larvae infected with *M. plutonius* after nine days oxytetracycline treatment

## Discussion

In this study *M. plutonius* bacteria have been isolated from (57) out of (69) samples of infected beehives with European foulbrood (EFB) disease. EFB affects mainly unsealed brood, killing honey bee larvae usually when they are 4–5 days old. Our result agree with several studies (3,10,11,12,13). The infected larva is coiled in its position, and when dies displaced in its cell, and twisted around the cell walls or stretch out. The color of the larvae change from pearly white to yellow, then brown, and finally when they decompose become grayish black. *M. plutonius* is found to lose its virulence quickly when sub cultured (in vitro) in laboratory media. This result agreed with (14, 15,16,17,18). The results show that there is elevation in mortality and infection rate of larvae from EFB. While after (OTC) treatment, the mortality infected rate is decreased. This result is agreed with (19) used oxytetracycline for treatment of European foulbrood in the UK. (20) reported that there are no *M. plutonius* strains resistant to OTC in Australia and all the 104 isolates test are sensitive to OTC. (21) test 80 isolates

from UK and demonstrate that all isolates are sensitive to OTC, also (22, 23, 24), in Canada (25) and India (26). Oxytetracycline hydrochloride (OTC) is a bacteriostatic antibiotic that inhibits the multiplication of *M. plutonius*. It is the only antibiotic approved for treatment of AFB and EFB. In both countries fumagillin is allow for use in the treatment of nosemosis. The same antibiotics used by beekeepers in Argentina. In addition, also a product containing sulfadimethoxine, trimethoprim, and oxytetracycline as pharmacologically active substances is approved in Argentina and eastern Scotland to be used against foulbrood and nosemosis (27, 28, 29, 30). In Conclusion: *M. plutonius* is a very exigent microorganism, and could be cultivated on a medium containing yeast extract. Oxytetracycline is effective in treatment of EFB and can cure the beehives within 9 days.

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