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**Rural Industries Research and  
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# **Oxytetracycline sensitivity of *Paenibacillus larvae*. subsp. *larvae* isolates**

**A report for the Rural Industries Research  
and Development Corporation**

by Michael Hornitzky

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# Foreword

American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae* subsp. *larvae*, is a major bacterial honey bee disease which causes significant economic loss to the beekeeping industry in Australia and around the world. Oxytetracycline hydrochloride (OTC) has been used to treat AFB for 4 decades. However, in recent years OTC-resistant strains have emerged in the United States of America, Canada and Argentina. Although in Australia OTC is only used in Tasmania to treat AFB it is important for the Australian beekeeping industry to know whether OTC-resistant *P. l. larvae* strains are in Australian bees and whether imported honey contains OTC-resistant *P. l. larvae*. The presence of OTC-resistant *P. l. larvae* in Australian bees will influence the choice of future control options for the control of AFB. It is also possible that OTC-resistant *P. l. larvae* may transfer this resistance to *M. pluton*, the cause of European foulbrood, another important bacterial disease of honey bees in Australia.

The aim of this study was to; (i) acquire a range of *P. l. larvae* from around Australia and determine the minimum inhibitory concentration (MIC) of OTC to these isolates, (ii) determine the MIC of OTC to *P. l. larvae* isolates obtained from imported honey and (iii) compare current OTC sensitivities to the MICs of *P. l. larvae* isolates collected in the late 1980s.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1200 research publications, forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry.

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**Tony Byrne**

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# Executive Summary

American foulbrood (AFB), caused by *Paenibacillus larvae* subsp. *larvae*, is considered to be the most important bacterial disease of honey bees in Australia. In many countries oxytetracycline hydrochloride (OTC) is used to treat the disease. On mainland Australia AFB is controlled by the incineration of infected hives or the irradiation of hive material from diseased hives. Tasmania is the only state which permits treatment with OTC.

In recent years OTC-resistant *P. l. larvae* have emerged in the United States of America, Canada and Argentina. There is no information on the OTC sensitivity of *P. l. larvae* in Australian bees and whether honey imported from overseas (Argentina) contains OTC-resistant *P. l. larvae*. This information is important as it has a bearing on future control options for bacterial honey bee diseases in Australia.

This study has demonstrated that *P. l. larvae* isolated from Australian sources are very sensitive to OTC and that no resistance to OTC appears to have developed over the past 15/16 years. Most isolates from imported honey had higher minimum inhibitory concentrations for OTC than Australian isolates but the difference was so minor that they would all still be considered to be very sensitive to OTC. This indicates that honey imported from Argentina has not been a significant source of OTC-resistant *P. l. larvae*.

# 1. Introduction

American foulbrood (AFB), caused by *Paenibacillus larvae* subsp. *larvae*, is a major bacterial honey bee disease which causes significant economic loss to the beekeeping industry in Australia and around the world. Oxytetracycline hydrochloride (OTC) has been used to treat AFB for 4 decades. However, in recent years OTC resistant strains have emerged in the United States of America, Canada and Argentina (Miyagi et al, 2000). Although in Australia OTC is only used in Tasmania to control AFB it is important for the Australian beekeeping industry to know whether OTC-resistant *P. l. larvae* strains are in Australian bees as the presence of such organisms in the Australian bee hives will influence the choice of future control options for the control of AFB.

It is also possible that OTC-resistant *P. l. larvae* may transfer this resistance to *Melissococcus pluton* (the cause of European foulbrood). OTC is the only antibiotic registered in Australia for use against EFB. The development of OTC-resistant *M. pluton* would have a severe impact on the profitability of bee farming in Australia and necessitate the introduction of an alternative antibiotic to treat EFB.

## 2. Objective

The aim of this study was; (i) to acquire a range of *P. l. larvae* from around Australia, (ii) to determine the minimum inhibitory concentration (MIC) of OTC to these isolates, (iii) to determine the MIC of OTC to isolates obtained from Argentinean honey and (iv) to compare current OTC sensitivities to the MICs of *P. l. larvae* isolates collected in the late 1980s.

## 3. Methodology

### 3.1 *Paenibacillus larvae* subsp. *larvae* isolates

*P. l. larvae* isolates were obtained by culture of honey samples and larval smears submitted by beekeepers in Australia or by veterinary laboratories in Queensland, Western Australia and Tasmania (Hornitzky and Anderson, 2002). Isolates were also cultured from imported honey collected from supermarket shelves and honey samples from individual Argentinean beekeepers provided by a honey packing plant. Isolates from the *P. l. larvae* culture collection held at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales were also used for comparative purposes to determine whether any OTC resistance had developed during the past 15 or 16 years.

### 3.2 Determining the minimum inhibitory concentration of OTC for *P. l. larvae* isolates

The MICs of OTC to *P. l. larvae* isolates were determined using the agar dilution method. *P. l. larvae* cultures which were initially cultured on 7% sheep blood agar (SBA) (Hornitzky and Clark, 1991) and incubated at 37°C for 2 days in an atmosphere containing 5% CO<sub>2</sub> were used as inocula.

Inocula were prepared by touching a single colony from a pure (second subculture) culture of *P. l. larvae* with a bacteriologists loop. A single line was then streaked on brain heart infusion agar plates (5 lines per plate). Plates were incubated as described previously.

Isolates were considered to be sensitive to a specific OTC concentration if their growth was markedly or completely inhibited by that concentration (Stokes and Ridgway, 1980).

## 4. Results

### 4.1 *Paenibacillus larvae* subsp. *larvae* isolates

Seventy nine *P. l. larvae* isolates were used in this study. These consisted of isolates from New South Wales, Queensland, Western Australia, Victoria, South Australia, Tasmania, imported honey purchased from supermarkets, imported honey from individual Argentinean beekeepers and Australian isolates collected in 1988/1989 (Table 1). These isolates were identified as *P. l. larvae* as described by Hornitzky and Anderson (2002).

### 4.2 The MIC of OTC for *P. l. larvae* isolates

Preliminary MIC assays indicated that the test isolates belonged to two groups; those that were sensitive to 0.1 µg/ml of OTC and those that were resistant to 0.1µg/ml. Following further preliminary studies the first group of isolates were then tested on two separate occasions with 0.01, 0.02, 0.03, 0.04 and 0.05 µg/ml of OTC. Those isolates resistant to 0.1µg/ml were tested on two separate occasions to 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µg/ml. Of the 79 isolates used in this study one isolate had a MIC of OTC of 0.03 µg/ml, 56 had a MIC of 0.04 µg/ml and seven had an MIC of 0.05 µg/ml. Four had a MIC of 0.5 µg/ml and 11 had a MIC of 0.6 µg/ml. All these 15 isolates except one were cultured from imported honey or honey which contained blends which also included imported honey. The one exception was an isolate cultured from a honey sample from a Victorian beekeeper (Table 1).

Table 1. Sensitivity of *P. l. larvae* isolates to OTC

Origin	No of isolates sensitive to OTC (µg/mL)					
	No of isolates	0.03	0.04	0.05	0.5	0.6
New South Wales	15	0	14	1	0	0
Queensland	16	1	15	0	0	0
Western Australia	7	0	7	0	0	0
Victoria	7	0	5	1	0	1
South Australia	3	0	3	0	0	0
Tasmania	4	0	4	0	0	0
Imported honey (ex supermarket)	13	0	6	0	1	6
Imported honey (ex specific beekeepers)	7	0	0	0	3	4
Old cultures (1988/89)	7	0	2	5	0	0
<b>Total</b>	<b>79</b>	<b>1</b>	<b>56</b>	<b>7</b>	<b>4</b>	<b>11</b>

## 5. Discussion

OTC has been used, for up to 40 years, for the treatment of AFB in a number of countries including the United States of America, Canada and Argentina. However, in recent years there has been an emergence of OTC-resistant *P. l. larvae* strains in these three countries. The degree of sensitivity/resistance to OTC reported by Miyagi et al (2000) has been quite variable ranging from >32.00 µg/ml for the American UCD P-MN spore isolate, to 10-15 µg/mL for Argentinian isolates to <1.00 µg/ml for the susceptible American strain (NRRL B-3650). However, there is no information on the sensitivity of Australian *P. l. larvae* to this antibiotic.

In this study all the isolates, except one, cultured from Australian brood or honey samples were sensitive to very low concentrations of OTC (from 0.03 to 0.05 µg/ml; Table 1). These isolates were of the same order of sensitivity as those in the late 1980s indicating that *P. l. larvae* has not developed any resistance over the past 15 years. All isolates cultured from the imported honey samples from beekeepers from Argentina were susceptible to OTC but at about 10 times the concentrations required to inhibit the growth of the Australian isolates. Despite the increased concentration of OTC required to inhibit the isolates from the Argentinean honey all these isolates would still be considered to be very sensitive to OTC. It has previously been reported that Australian isolates of *Melissococcus pluton* (the cause of European foulbrood) are sensitive to 1 or 2 µg/ml (Hornitzky and Smith, 1999). This is about 4 times the concentration required to inhibit Argentinean *P. l. larvae* isolates detected in this study.

There was one isolate cultured from a Victorian honey sample which had an MIC equivalent to those cultured from all the honey samples submitted from the Argentinean beekeepers. Although this isolate is considered to be sensitive to the OTC it indicates that there may be transfer of *P. l. larvae* from imported honey to Australian honey bee colonies.

During this study it was noticed that as the more the *P. l. larvae* isolates were subcultured the more quickly they became adapted to increased concentrations of OTC. To avoid the artificial detection of OTC-resistant *P. l. larvae* isolates the test cultures were not subcultured more than twice after culture from the stock culture agar slope. The marginally increased resistance of the 1988/89 isolates to OTC may have been a function of their increased subculture rates although there are no records of how many times they had been subcultured before the assays in this study were undertaken.

In summary this snapshot study has demonstrated that *P. l. larvae* isolates of Australian origin are very sensitive to OTC. Isolates cultured from honey imported from Argentina are also sensitive to OTC but at a level approximately ten fold above the sensitivity determined for Australian isolates. The single *P. l. larvae* isolate with a MIC of 0.6 µg/ml cultured from a Victorian beekeeper's honey sample suggests that an Argentinean *P. l. larvae* strain has infected an Australian honey bee colony. It would be useful to continue monitoring of this type to determine if the OTC sensitivity of *P. l. larvae* in Australian honey bee colonies changes.

## 6. Recommendations

That *P. l. larvae* isolates from Australian bees and imported honey continue to be monitored for their sensitivity to OTC.

That imported honey samples be cultured for *M. pluton* (the cause of EFB) to determine whether resistant *M. pluton* strains have been imported into Australia.

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