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Preliminary investigations into possible resistance to oxytetracycline in *Melissococcus plutonius*, a pathogen of honeybee larvae

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ABSTRACT

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Aims: To investigate the occurrence of oxytetracycline (OTC) resistance in *Melissococcus plutonius*, which causes European foulbrood in honeybee colonies.

Methods and Results: Strains of *M. plutonius* were isolated from diseased colonies in England and Wales and tested for resistance to OTC. The minimum inhibitory concentration (MIC) of OTC was also determined for selected isolates. No resistance to the antibiotic was found in any isolate and the average MIC was found to be 3·9 μg ml^{-1}. *Melissococcus plutonius* was found to be susceptible to both chlortetracycline and tetracycline.

Conclusions: No resistance to OTC was found in *M. plutonius*.

Significance and Impact of the Study: This study demonstrated that OTC can continue to be used to treat European foulbrood and that resistance may not explain why some treatments fail.

Keywords: *Apis mellifera* L., European foulbrood, *Melissococcus plutonius*, oxytetracycline resistance.

INTRODUCTION

Honeybees suffer from relatively few diseases, but those that do occur are often very serious. One of the major diseases affecting larvae is European foulbrood (EFB). The condition is thought to be caused by a bacterial pathogen, *Melissococcus plutonius* (formerly *M. pluton*), which is ingested by the larva during feeding by adults (Bailey and Locher 1968; Bailey 1983). The bacterium resides in the gut and competes with the larva for food. If food is plentiful, both larva and bacteria will survive. However, when food is less available, the bacteria will scavenge the food, causing the larva to starve and die (Bailey 1960). The aetiology is further complicated as there are several other bacteria associated with the disease, including *Paenibacillus alvei*, *Brevibacillus laterosporus* and *Enterococcus faecalis*. These are thought to be secondary saprophytes that multiply on the dead larva, rather than actual pathogens (Bailey 1963; Alippi 1991). However, the progression of the disease is not fully understood and these bacteria may play a further role.

If the infection is light, affecting few larvae within a colony, the disease can be treated with the bacteriostatic antibiotic oxytetracycline (OTC), which prevents multiplication of the bacteria. This antibiotic has been used in the UK since the late 1960s to treat EFB and, in some countries, is used against another honeybee disease, American foulbrood (AFB), caused by *P. larvae* subsp. *larvae*. Despite the similarity of their names, the two diseases are not related. Katznelson *et al.* (1952) found that OTC was effective in treating colonies with EFB. American beekeepers have used the antibiotic as a treatment and prophylactic measure against both foulbrood diseases since the 1950s (Moeller 1978; Lehnert and Shimanuki 1980; Hoopingarner and Nelson 1987; Kochansky 2000). Resistance of *P. larvae* subsp. *larvae* to OTC has recently been reported in Argentina and the USA (Alippi 2000; Miyagi *et al.* 2000). In the UK, colonies with AFB are destroyed as the disease is highly virulent and treatment can mask signs of EFB (Thompson and Brown 1999). Since *M. plutonius* has been exposed to OTC for a long period, it might be expected that

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there would be some resistance to the antibiotic. Many bacteria develop resistance to antibacterial agents to which they are commonly exposed, as this exerts a high selective pressure. Oxytetracycline resistance may explain why some treatments with the antibiotic are unsuccessful (Thompson and Brown 2001). Thus, the occurrence of OTC resistance in isolates of *M. plutonius* from honeybee colonies affected with EFB was investigated.

MATERIALS AND METHODS

Isolation and culture of *Melissococcus plutonius*

Larval samples affected with EFB are sent to the National Bee Unit laboratory as part of the Department for Environment, Food and Rural Affairs (DEFRA) and National Assembly for Wales Agriculture Department (NAWAD) statutory bee health inspection service. At the laboratory, samples with clinical signs of disease are examined for the presence of the causative agents, thus confirming the disease. Eighty samples of EFB-infected larvae were chosen randomly for isolation of *M. plutonius* and subsequent antibiotic resistance testing. Each sample was mixed with 1 ml sterile saline (0·85% NaCl) in a sterile Eppendorf tube. The resulting watery suspension was streaked out onto SYPG agar (Bailey and Ball 1991) (containing (g l^{-1})): KH_2PO_4, 13·5; yeast extract, 10; soluble starch, 10; cysteine, 1; glucose, 10 and agar (no. 1; Oxoid), 10. Chemicals were obtained from Fisher Scientific (Loughborough, UK) or Oxoid. Starch was dissolved completely in hot distilled water before addition to the medium and the final medium adjusted to pH 6·6 with 5 mol l^{-1} KOH. Once inoculated, plates were transferred to an anaerobic jar, into which was inserted an Anaerogen pouch (Oxoid). This produced an anaerobic atmosphere, allowing growth of *M. plutonius*, a microaerophilic organism that requires a small amount of carbon dioxide for optimal growth. Resazurin strips were used to indicate anaerobicity. Plates were incubated for at least 3 d until growth was evident. Colonies were Gram-stained to confirm that they were *M. plutonius*. The organism shows variable colony morphology in culture but usually grows as small (about 1–2 mm) greyish-white round colonies. It was important to ensure that the growth seen was not of a related organism, *E. faecalis*, which is sometimes found in colonies with EFB and is similar in appearance to *M. plutonius*. This bacterium will grow aerobically and so is easily distinguishable from *M. plutonius*.

Cultures for antibiotic resistance testing

Once isolated, cultures were subcultured until pure growth was obtained. Bacteria were freshly grown and harvested from SYPG agar by washing with 1 ml sterile saline. Several colonies were harvested to give a consistent cell suspension.

Resistance testing

Isolates of *M. plutonius* were tested using a disc diffusion assay with antibiotic discs containing 30 μg OTC (Oxoid). Plates were divided into three sections, with a different isolate inoculated onto each, and an OTC disc placed between each section; every plate was duplicated. The plates were then incubated anaerobically at 34 °C for 3–4 d.

The degree of resistance was taken as the radius of the zone of inhibition (referred to as 'inhibition distance') around the OTC disc. The measurements were taken from the edge of the OTC disc to the edge of the bacterial growth. Two measurements were taken from each of the two discs for each isolate; thus, four measurements were recorded for each isolate, from which the average inhibition distance was calculated.

Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of OTC for *M. plutonius* was determined using an agar plate method. Decreasing concentrations of antibiotic were used to determine the lowest concentration at which growth could occur.

The concentrations of OTC were made using a stock solution of 0·1 g 95% OTC in 10 ml distilled water. The 10-ml solution was then sterilized using a syringe-driven filter unit with a 0·22-μm pore size. Appropriate amounts of the antibiotic stock solution were added to cooled (but still molten) agar, which was then poured into Petri dishes.

Twelve isolates of *M. plutonius*, taken from different locations in England and Wales, were tested in duplicate and incubated at 34 °C for 3 d. The initial plates contained OTC at concentrations of 2, 4, 6 and 8 μg ml^{-1} and further plates were used with a narrowing range of concentrations to find the MIC.

Susceptibility to related antibiotics

Melissococcus plutonius was tested for susceptibility to antibiotics related to OTC. The procedure was as used for OTC resistance testing, except that one isolate of *M. plutonius* was spread across a whole plate and the discs used contained different antibiotics (chlortetracycline and tetracycline). Chlortetracycline was made up as a stock solution and placed onto blank filter discs to give 30 μg $disc^{-1}$ and tetracycline discs (30 μg $disc^{-1}$) were obtained from Oxoid. Another organism associated with

EFB, *B. laterosporus*, was tested at the same time to monitor susceptibility in this organism. The same procedure was used for this organism, but it was grown on nutrient agar (Oxoid) under aerobic conditions at 34 °C.

RESULTS

Isolation and culture of *Melissococcus plutonius*

Isolates of *M. plutonius* were obtained from infected larvae and subculture led to pure cultures, which were tested for antibiotic resistance.

Resistance testing

Data for 80 isolates are shown in Fig. 1 which shows the number of isolates and average inhibition distance.

The lowest inhibition distance for an isolate was 18·5 mm and the highest 31 mm. Although there was a wide range, the majority of isolates had inhibition distances greater than 25 mm with 21 (26·3%) isolates having distances smaller than this. Over half of all isolates tested showed an average inhibition distance greater than 28·5 mm.

Minimum inhibitory concentration determination

The results for the isolates of *M. plutonius* are shown in Fig. 2 which shows the mean value of the lowest OTC concentration at which growth was prevented.

The average concentration of OTC that inhibited growth was 3·9 (± 0·3) μg ml^{-1}. Only two isolates differed between replicates, and this was by just 0·1 μg ml^{-1} in both cases.

Susceptibility to related antibiotics

The results for the susceptibility of both *M. plutonius* and *B. laterosporus* to all three antibiotics tested are shown in Table 1.

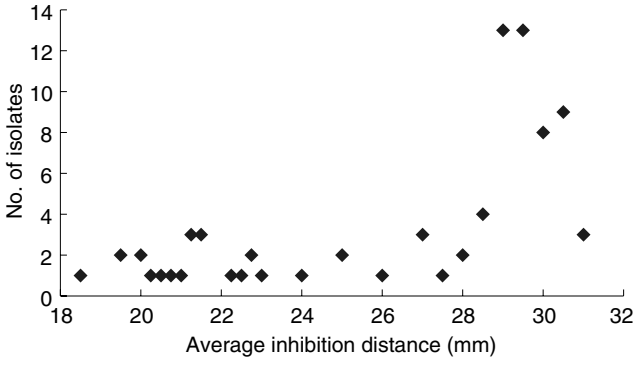

Fig. 1 Average inhibition distance for *Melissococcus plutonius* isolates challenged with oxytetracycline

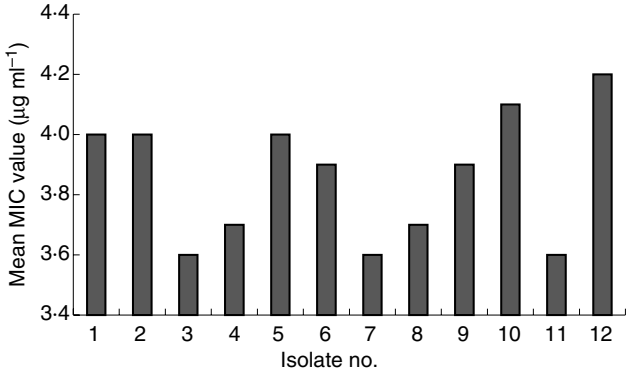

Fig. 2 Mean minimum inhibitory concentration (MIC) values for selected isolates of *Melissococcus plutonius*

Melissococcus plutonius was very susceptible to all of the antibiotics tested. *Brevibacillus laterosporus* showed a more varied response, with a higher susceptibility to OTC than the other antibiotics.

DISCUSSION

Resistance to OTC in *M. plutonius* may explain why some treatments of EFB with this antibiotic do not work. If the bacteria present are not susceptible, the antibiotic will have no effect on the infection. However, this study showed that all isolates tested were susceptible to OTC. The disc diffusion test used here was intended to be the primary method of detecting any resistance to the antibiotic, with any deviation from the norm followed up with further tests. However, these further tests were unnecessary as it was evident that no isolate tested showed apparent resistance to OTC, as indicated by correlating the average MIC values (which were similar to values for susceptible bacteria in Australia) with the average inhibition zone values.

There are few reports of *M. plutonius* being tested for antibiotic resistance. A survey carried out in Australia found that all 104 isolates tested were still highly sensitive to OTC, with MICs of between 1 and 2 μg ml^{-1} (Hornitzky and Smith 1999). Another interesting factor is that an earlier study conducted in Australia in 1988 (Hornitzky *et al*. 1988) found the MIC to be 3·8 μg ml^{-1}, not dissimilar to the level found in the current study, indicating UK isolates to be susceptible. This behaviour may reflect some intrinsic properties of the organism. *Melissococcus plutonius* has very similar DNA profiles irrespective of its geographical origin (Djordjevic *et al*. 1999) and the phenotypic responses shown here support these genotypic data.

There is no evidence that *M. plutonius* has developed resistance to OTC in the UK. Indeed, some of the isolates included in the current study were from recurrences of

Table 1 Average inhibition distance of bee-associated bacteria to selected antibiotics

Organism	Average inhibition distance (mm)		
	Oxytetracycline	Tetracycline	Chlortetracycline
Brevibacillus laterosporus	10·25	6·5	8·25
Melissococcus plutonius	20·75	19·50	20·75

infection earlier in the season and these showed the same level of susceptibility to OTC as other isolates. These isolates were most likely to show resistance as treatment with OTC had been unsuccessful in eliminating EFB from colonies.

There has been great interest in the antibiotic susceptibility of bacteria pathogenic to honeybees in recent years, due to the discovery of OTC-resistant *P. larvae* subsp. *larvae* (with MICs of > 32 $\mu g\ ml^{-1}$) from colonies where OTC had failed to control AFB. Susceptible strains were quoted as having MICs of < 5 $\mu g\ ml^{-1}$ (Miyagi *et al*. 2000) which, by comparison, would indicate that all of the strains of *M. plutonius* tested in the current study are susceptible. Resistance has so far been found in North and South America, where prophylactic treatment with OTC is commonplace and unregulated; this approach may have led to resistance. Shimanuki and Knox (1994) found no difference in susceptibility to OTC between American strains of *P. larvae* subsp. *larvae* isolated before the use of OTC was widespread and contemporary strains. Resistance may, therefore, be a recent phenomenon. In the USA, other antibiotics are being screened for use as AFB treatments, but this may lead to similar problems in the future (Feldlaufer *et al*. 2001; Kochansky *et al*. 2001). A different approach has been used in New Zealand where husbandry methods are used to control AFB, similar to the situation in the UK (Van Eaton 2000).

The mechanism of OTC resistance in *P. larvae* subsp. *larvae* has not yet been elucidated. Resistance to tetracyclines has been disseminated to many other genera, both Gram-negative and -positive, primarily by plasmid transfer or transposons (Adams *et al*. 1998; Schnabel and Jones 1999; Rhodes *et al*. 2000). The mechanisms of resistance are not fully understood, but are due either to active efflux systems removing the antibiotic from the cytoplasm or ribosomal modification protecting against the effects of tetracyclines (Roberts 1996).

Antibiotic use in the UK is strictly controlled and it is illegal for beekeepers to apply OTC themselves or to use it to treat AFB. It is, perhaps, these strict controls that have led to the continued susceptibility of the organism. Another factor is that if a treatment is unsuccessful, i.e. there is EFB recurrence in the same colony within the season, the colony will be destroyed (Thompson and Brown 1999). This may mean that any bacteria that evolve resistance to OTC are destroyed before they can be disseminated to other colonies. However, isolates from colonies with recurrences of infection were found to be no more resistant to OTC when compared with isolates from 'primary' infections. *Brevibacillus laterosporus* appears to have intrinsic resistance to the antibiotics tested, with relatively low inhibition distances in comparison to *M. plutonius*. Although it is possible that resistance determinants could be transferred to pathogenic bacteria within the same colony, any recurrence of disease will lead to colony destruction, thus limiting the impact of this risk.

Oxytetracycline could continue to be used to treat EFB for the foreseeable future, although other methods, including husbandry techniques which would reduce reliance on antibiotics, are under investigation. The resistance screening programme will continue in order to anticipate potential problems, similar to the successful scheme introduced to allow early detection of pyrethroid resistance in *Varroa destructor* (Thompson *et al*. 2002). Isolates of *P. larvae* subsp. *larvae* will also be assayed to determine whether there is any resistance to OTC in the UK.

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