LIMITS OF CHEMOTHERAPY IN BEEKEEPING: DEVELOPMENT OF RESISTANCE AND THE PROBLEM OF RESIDUES

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Abstract

The development of resistance in honeybee pests and diseases means that conventional drug treatments are becoming less effective. Also, chemical residues in bee products are a serious problem for beekeepers. The main diseases that affect honeybees worldwide and for which chemical treatments are used are the bacterial infections American (AFB) and European (EFB) foulbrood, and the parasitic mite, Varroa destructor. However, due to the development of resistant strains of AFB and varroa and the problem of residues (antibiotics in honey and acaricides in wax), the limits of chemotherapy have become evident. Resistance describes a population of organisms (insects, mites or bacteria), that is no longer controlled by what formerly had been a lethal dose of a pest control chemical. It is a well known and widespread evolutionary phenomenon and depends on the presence of heritable variability. In most populations, however, the presence of resistant genotypes is due to immigration from other populations (gene flow). The genes for resistance must already be present in the population before exposure to the pesticide begins. The mutations that give rise to resistance occur independently from the exposure, as they are chance events.

Introduction

Bees are often bred for behavior traits, which are not in harmony with their natural survival strategies. As a consequence, they function less efficiently under nutritional stress conditions and their immune system can be compromised by toxic elements in the environment. The beekeepers’ task therefore is to recognize stress symptoms in domestic colonies and to assist them through difficult times. In many cases when the beekeepers see their livelihoods threatened by disease in the bee colonies they use drugs as a first option – if they can afford them and they are locally available. In the short term this option is often the most convenient economically and usually does not require in-depth knowledge of the biology of the pest.

Often reference is made to resistance ‘developing’ in a process that takes place at different locations, as the result of changes at many loci, whereas in fact resistant individuals have existed all along. Genetic variation, mutations and recombination alter the genetic make-up of the host and pathogen continuously and genetic changes in either organism may help one organism deal with the other, while some changes reduce an organism’s ability to cope.

In most cases resistance is a monofactorial trait. In the field, polygenic resistance can develop but it would develop and spread more slowly. Generally, a treatment with a given chemical removes nearly all the nonresistant individuals at first, but leaves the resistant individuals alive. If the number of survivors containing the genes for resistance is high enough, then resistance in the population will develop more quickly when the chemical treatment is used. The only effect that we can have on this process is to speed it up or to slow it down. If every new generation of the pest is exposed to the pesticide, then resistance will develop at a faster rate. For this reason, timing and dosage of any pesticide application is important in the development of resistance and abuse, under use or misuse of chemotherapeutic substances must be avoided.
Resistance to antibiotics

Use of antibiotics to control AFB

American foulbrood (AFB) is a virulent brood disease and is caused by *Paenibacillus larvae* larvae, which has a long-lived, resistant spore that can remain dormant for years in combs and honey. AFB is spread by the exchange of infected honey and combs among colonies, either by the beekeeper or by robber bees. If no measures are taken by the beekeeper the colony is very likely to be destroyed by the infection, thus becoming a source of contagion for the whole apiary.

The most commonly used antibiotic for the control of brood diseases is oxytetracycline (OTC) (trade name Terramycin or TM25), a broad-spectrum bacteriostatic antibiotic that affects the 16S and 18S rRNAs genes of both rampositive and Gram-negative bacteria. In the USA, AFB has been treated with this single registered antibiotic since the 1970s.

Macrocyclic lactone tylosin, (which has current US Food and Drug Administration approvals for agricultural uses) may soon gain approval for the control of AFB. This antibiotic inhibits ribosomal protein synthesis and has recently shown a good efficacy, while other substances failed to cure AFB in colonies with a high level of spores. The β-lactams (penicillins and cephalosporins), while active in vitro, are apparently not effective in the field. No antibiotic is capable of acting through the thickened wall of the bacillus spore and for this reason antibiotics are said to ‘mask’ the infection for the whole duration of their use; usually the disease reappears when the treatment is interrupted because the spores remain viable for several decades or longer.

Use of antibiotics to control EFB

OTC is an excellent treatment for EFB as ingestion of the antibiotic by nurse bees results in secretion of OTC-containing food fed to the larvae. In Australia it is the only treatment licensed for use in controlling EFB; in the UK, where light EFB infections are frequently discovered, treatment of the colony with OTC can be administered at the discretion of a bee inspector. In Canada (BC) EFB is easily controlled with standard antibiotic treatments. In other countries, administration of OTC is permitted as a preventive as well as a curative treatment.

To date no OTC resistance by *Melissococcus plutonius*, the causative agent of EFB, has been reported, but it is also possible that OTC-resistant *P. l. larvae* may transfer this resistance to *M. plutonius*.

Recently, ampicillin and amoxicillin have been successfully tested for use in controlling EFB. *M. plutonius* was found to be highly susceptible to β-lactam antibiotics and they degrade at a much higher rate than the other test antibiotics. Ampicillin and amoxicillin however are widely used as human antibiotics and as such would face greater scrutiny for new agricultural uses.

OTC resistance in *P. l. larvae*

Resistance to OTC has been observed in *P. l. larvae* isolates from several countries (Argentina, New Zealand, USA, Canada, Italy). The first question is related to the presence of OTC-resistant bacteria in geographically distant populations: does this represent an independent origin of the trait or a single origin spread by transport of bees and hive materials? Considering the commerce among countries (USA, Argentina, Australia) involved with the problem, it is tempting to assume a single origin of OTC resistance followed by spreading of this trait through the movement of bees.
Using PCR analysis with primers that anneal to dispersed repetitive bacteria sequences, Alippi generated genomic fingerprints of a collection of 100 *P. l. larvae* Argentinean isolates; the strains were grouped in three clusters using BOX-primers. In relation to their response to OTC, the author found a 48% resistance within the collection of 91 *P. l. larvae* strains; for this group, the values of minimal inhibitory concentration (MIC) were 10–15 µg/ml, while the susceptible ones presented MIC values under 5 µg/ml. Other isolates from France, Italy, New Zealand, Sweden, USA, Poland, Czech Republic and Germany were susceptible, with MIC values under 5 µg/ml. Miyagi reported a variable degree of sensitivity/resistance to OTC, ranging from > 32 µg/ml for the American (Minesota) spore isolate, to 10–15 µg/ml for Argentinean isolates, and < 1 µg/ml for other susceptible American strains. Hornitzky, studying isolates cultured from Australian brood or honey samples found that all except one were sensitive to very low concentrations of OTC (from 0.03 to 0.05 µg/ml). These isolates were of the same order of sensitivity to those reported 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 Argentinean honey samples 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.

**Sulfathiazole resistance**

Sulphonamides suffer from increased bacterial resistance and high toxicity. In the 1990s all the isolates tested in Germany were classified as resistant to this class of drugs. Sulfathiazole lost its FDA approval about 30 years ago. Nevertheless, residue levels in honey from the EU market seem to indicate that the use of this substance is still widespread.

**Mechanisms of antibiotic resistance**

Bacteria exhibit many mechanisms to protect themselves from antibiotics. Antibiotic modification is the best known: the resistant bacteria retain the same sensitive target as the antibiotic-sensitive strain, but the antibiotic is prevented from reaching it. This happens, for example, with β-lactamases which enzymatically cleave the four membered β-lactam ring, rendering the antibiotic inactive (e.g. ampicillin and amoxicillin, candidates for the control of EFB in Australia). β- lactamases are widespread
among many bacterial species (both Gram positive and Gram negative) and exhibit varying degrees of inhibition by β-lactamase inhibitors.

Resistance genes and mutation rate

Antibiotic resistance can be achieved by horizontal acquisition of resistance genes, carried by transposons or plasmids, by recombination of foreign DNA into the chromosome, or by mutation in different chromosomal loci (fig.1). Some transposons in bacteria carry – in addition to the gene for transposase – genes for one or more (usually more) proteins imparting resistance to antibiotics. When such a transposon is incorporated into a plasmid, it can leave the host cell and move to another: this is the way that the alarming phenomenon of multidrug antibiotic resistance spreads so rapidly. In the genus *Bacillus* the number of observed nucleotide changes due to recombination in natural populations of bacteria has been found to range from approximately equal to the number of changes due to mutation. In studies of molecular evolutionary biology the term ‘mutation rate’ is applied to estimations of the rate of mutation per nucleotide, per locus or eventually for the whole genome, and selective favourable, unfavourable, or neutral mutations are considered. Differing with this concept, the ‘frequency of mutation’ measures all the mutants present in a given population, irrespective of whether the mutation events occurred early or late during the growth of the populations. In this respect, the frequency of mutants is a cross section of the bacterial population at a given time and reflects not only the mutation rate but also the history of the population before selection is applied. In the case of antibiotic resistance, the mutation rate is frequently defined as the *in vitro* frequency at which detectable mutants arise in a bacterial population in the presence of a given antibiotic concentration.

Resistance to acaricides

Although effective acaricides to control *Varroa destructor* are available, the mite has a damaging effect on beekeeping with most *Apis mellifera* subspecies. Mite infestation constitutes a continuous risk, which may lead to weakness or loss of colonies as soon as failure of the employed treatment occurs.

Resistance to acaricides is a serious problem in chemotherapy for *V. destructor* and can cause disastrous colony losses if control of the mite relies on ineffective treatments.

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**Fig. 2. Strategies for the control of American foulbrood.**
Fluvalinate resistance
Concerning the situation in European countries, the temporal spread of mite resistance suggests that it has arisen only once or twice and that the major cause for the rapid spread of resistant mites is the movement of bee colonies by beekeepers. Two different mechanisms of resistance have been suggested for fluvalinate: one concerns the increase in the levels of detoxification enzymes, such as mono-oxygenases in mite populations in Europe and Israel (mono-oxygenases are the enzymes that make fluvalinate almost harmless to the bee); the other concerns a reduced target-site (sodium channel) sensitivity to pyrethroids in the nervous system (knockdown resistance: a mechanism reported for many insect pests) as was found in the Florida and Michigan mite populations. These studies suggest that pyrethroid resistance has arisen twice. In the ‘Italian’ fluvalinate-resistant mite strain, cross-resistance between fluvalinate and two closely related pyrethroids – flumethrin and acrinathrin – was found. The presence of cross-resistance is not unexpected due to the similarity in the molecules of the active ingredient. Thus, these pyrethroids cannot be used as alternatives to fluvalinate where Apistan (tau-fluvalinate) fails in controlling varroa. Mites resistant to fluvalinate do not show an increased tolerance to coumaphos, but it is not known whether resistance to coumaphos increases tolerance to fluvalinate.

Coumaphos resistance
Organophosphate and carbamate insecticides irreversibly inhibit acetylcholinesterase, causing death of insects. Resistance-modified acetylcholinesterases (AChEs) have been described in many species and sequencing of their genes allowed several point mutations to be described. Some *V. destructor* populations are resistant to coumaphos, a widely used organophosphate acaricide. The first reports concerning a modest but significant increase in the mites’ tolerance to coumaphos in some areas of northern Italy date back to 1997 (fig. 3). In 1999, populations of *V. destructor* with a LC₅₀ increased by about 20-times were detected in northern Italy. Later, bee losses due to failure in the control of the mite with coumaphos were observed in other regions. It is known that a high-dose strategy against varroa is inadequate because the mites are highly inbred with an excess of homozygotes. More recently, coumaphos resistant populations were also reported in Switzerland and in the USA only a few years after the commercialization of a gradual-release coumaphos-based anti-varroa treatment product.

![Fig. 3. Countries where resistant mites (coumaphos: red arrow; amitraz; blue arrow) have been reported.](image)

Amitraz resistance
In Croatia lack of efficacy of treatments with amitraz was reported and it seems that the most likely explanation is the presence of resistant mites. More recently, inefficacy in the field of an amitraz-based product has been observed in France and in the some states in the USA. In laboratory assays, mites from the USA showed an increased tolerance to amitraz (fig. 3).

**Resistance, fitness and reversion**

In some cases, possessing an unusual characteristic which makes an individual resistant can result in a less efficient metabolic or reproductive capacity compared to the average. Populations of resistant arthropods demonstrate lower fitness than susceptible ones. The reduction in fitness could be due to the production of a large amount of detoxifying enzymes which are useless, if not detrimental, in the absence of pesticides or in unbalanced physiological processes associated with resistance. The reduction in fitness sometimes produces individuals with smaller body size and increased asymmetry. A decrease in fitness in the order of only a few percent per generation would produce an appreciable disadvantage over a year, since several generations of the mite take place in this period. On the other hand, the selection pressure increases dramatically when the efficacy of treatments approaches 100% and the same acaricide is used repeatedly or for a prolonged period, correspondingly reducing the effect of any disadvantage of the resistant strains. To avoid this phenomenon, in Italy, many beekeepers treat the hives for the mite with a combination of essential oils (commonly thymol) at the end of the summer, with a single treatment of oxalic acid in late autumn. Compared to susceptible mites, resistant mites generally have a lower fitness; this causes a progressive decrease in the frequency of resistant genotypes when the selective pressure due to the chemical treatments is removed. This phenomenon is called, not very appropriately, reversion. In the case of *V. destructor*, the frequency of fluvalinate-resistant individuals decreases very slowly (a factor 10 decrease in 3 years). A progressive decline in the percentage of resistant mites was observed in Friuli (NE Italy), a region where no pyrethroids were applied for some years, suggesting that the disadvantage associated with the resistance can counterbalance the selection of fluvalinate resistant mites if treatments with this substance are used once in about four years. Reduction of fitness is in any case quite small, as always happens when resistance is due to mono-oxygenizes.

![Fig. 4. Residues of acaricides in Italian wax from mid 90s to 2003 (Istituto Nazionale di Apicoltura, Bologna, Italy).](image-url)
Resistance management
The only way to halt the development of resistance to a certain product is by interrupting its use in the control strategy, and possibly that of all related compounds, for a certain period of time.

AFB
In the control of AFB, alternatives to chemically synthesized antibiotics do exist and are viable.

Management of the colonies
In countries in which the use of antibiotics is the only strategy against infective brood diseases of the honey bee colony, there is an urgent need to identify new control approaches (fig. 2).

In a long-term strategy, sanitary practices and common sense are the best tools beekeepers can use to prevent the spread of AFB. Recognizing disease symptoms in honey bee colonies is an essential part of good beekeeping management. Early detection allows for prompt remedial action and helps in preventing serious disease outbreaks and economic losses. Regular inspection of the brood combs, being thoroughly familiar with visual detection of brood diseases, reducing the exchange of hive equipment between hives and apiaries, replacing 20% of all brood frames each year, burning all the colonies with evident disease symptoms (or shaking bees onto foundations if the disease is limited to a few brood cells) are the most recommended practices to drastically reduce AFB incidence in apiaries.

BIBLIOGRAPHY