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Veterinary Microbiology 109 (2005) 47–55
veterinary microbiology
www.elsevier.com/locate/vetmic

In vitro and in vivo susceptibility of the honeybee bacterial pathogen *Paenibacillus larvae* subsp. *larvae* to the antibiotic tylosin

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Received 26 November 2004; received in revised form 2 March 2005; accepted 2 March 2005

Abstract

The minimal inhibitory concentrations (MICs) of tylosin were determined to 67 strains of *Paenibacillus larvae* subsp. *larvae,* the causal agent of American Foulbrood (AFB) disease, from different geographical origins. MIC values obtained ranged from 0.0078 to 0.5 μg/ml. These very low values imply that no resistance to tylosin was found in any isolate of the Foulbrood pathogen.

The measurement of diseased larvae with AFB-clinical symptoms in three different field studies demonstrated that tylosin treatment could be effective in vivo. No negative effects in colonies were noted at any dosage rates or forms of application. These studies demonstrate that tylosin, as tartrate, can be used to treat AFB in honeybee colonies.

Keywords: American Foulbrood; Tylosin; *Paenibacillus larva*e subsp. *larvae*

1. Introduction

American Foulbrood (AFB) disease caused by the spore-forming bacterium *Paenibacillus larva*e subsp. *larvae* (*P.l. larvae*) (Heyndrickx et al., 1996) (formerly *Bacillus larvae*) is a cosmopolitan disease of bacterial origin affecting the larval and pupal stages of honeybees (*Apis mellifera* L.) (White, 1920; Shimanuki, 1990). Diseased individuals turn brown, then black, and the resultant mass becoming a hard scale of material deposited on the side of the cell. AFB is one of the few bee diseases capable of killing a colony, and has unique problems for prevention and control because the spores can remain viable for long periods

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doi:10.1016/j.vetmic.2005.03.008

of time and survive environmental adversities (Matheson and Reid, 1992). The disease is highly contagious and if undetected can kill a colony and spread to others within the same apiary or to another apiary nearby by robbing and exchanging of brood combs, the main sources of contamination.

In areas where disease incidence is high, antibiotic treatments appear as an alternative to the burning of diseased bee colonies. Currently, the only antibiotic approved for prevention and control of AFB is oxytetracycline, however, there is evidence of oxytetracycline-resistant isolates of *P.l. larvae* in certain areas of the USA, Canada and Argentina (Alippi, 2000; Colter, 2000; Evans, 2003; Miyagi et al., 2000).

The antibiotic tylosin, as tartrate, has been shown to be an alternative to oxytetracycline for the control of AFB in several studies (Alippi et al., 1999; Elzen et al., 2002a,b; Hitchcock et al., 1970; Moffett et al., 1970; Peng et al., 1996). Tylosin is virtually non-toxic to adult honeybees (Alippi et al., 1999) and less toxic than oxytetracycline to honeybee larvae (Peng et al., 1992, 1996).

The purposes of the present work were to evaluate the susceptibility of 67 strains of *P.l. larvae* from diverse geographical areas to tylosin by determining their minimal inhibitory concentrations (MIC), since the information about their efficacy in vitro is quite limited (Alippi, 1994; Kochanski et al., 2001; Okayama et al., 1996) and to determine the response of colonies with clinical signs of AFB to tylosin at different doses and forms of application in order to determine the most effective dose for disease control and lack of recurrence of the disease.

2. Materials and methods

2.1. Bacterial strains

The 67 *P.l. larvae* strains from diverse geographical origins used in this study are listed in Table 1. For the isolation of *P.l. larvae* strains from larvae affected by AFB and from honey samples, previously described techniques were employed (Alippi, 1995; Alippi and Aguilar, 1998; Alippi et al., 2004). In addition, reference strains from culture collections were included, and also strains of *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* used as reference standards for quality control ranges of MICs were provided by Instituto Malbrán Collection, Instituto Malbrán, Buenos Aires, Argentina (Table 1).

2.2. Determination of minimal inhibitory concentrations of tylosin

As there is no NCCLS recommendation for the determination of MICs of *P.l. larvae*, a method developed for this species was used based on the NCCLS standard for Bacteria isolated from animals (NCCLS, 1999). Minimal inhibitory concentrations of tylosin tartrate (Sigma®) were determined by the agar dilution method using MYPGP (Dingman and Stahly, 1983) as basal medium because *P.l. larvae* is not able to growth on Muller–Hinton agar. Tylosin concentrations tested were made using a stock solution of 5000 μg/ml in distilled water and sterilized using a syringe-driven filter unit with a 0.22 μm pore size. Appropriate dilutions were made in sterile distilled water and stored at −20 °C until used. The medium was maintained at 45 °C until the antibiotic solutions were incorporated and 25 ml of culture medium were poured onto each Petri plate of 90 mm in diameter. Increasing concentrations of tylosin tested were: 0.0039, 0.0078, 0.015, 0.03125, 0.0625, 0.125, 0.5, 1, 2, 4, 8, 16 and 32 μg/ml of medium. For the controls, MYPGP agar without antibiotic was employed.

Vegetative cells of each *P.l. larvae* strain grown on MYPGP agar for 48 h of incubation at 37 °C were suspended in sterile distilled water and adjusted to approximately 2.87×10^8 cells/ml ($A_{620\ nm} = 0.388$, equivalent to a Mc Farland standard of 1). The different bacterial strains were layered by using an automatic micropipette to place drops of 10 μl each over the surface of the solidified culture medium containing each antibiotic concentration. Ten replications were tested for each strain. The inoculated plates were examined for growth after 48 h of incubation at 37 °C. The lowest concentration of antibiotic preventing growth was defined as the MIC (complete inhibition of bacterial growth on the test plates, disregarding a single colony of faint haze caused by inoculum).

In the case of *P. aeruginosa, E. coli* and *S. aureus* used as reference standards, the technique outlined by the NCCLS for each species (NCCLS, 1999) was followed, with the only difference that MYPGP agar was used as basal medium.

Table 1
Minimal inhibitory concentration values (MICs) (μg/ml) for selected isolates and designation and origin of the bacterial strains used in this study

Strain	Other designation	Geographical origin	Source	Year	MIC
Paenibacillus larvae subsp. *larvae*					
PL3		Concordia, Entre Ríos, Argentina[a]	Diseased larvae	1992	0.0625
PL7		Pigüe, Buenos Aires, Argentina[a]	Diseased larvae	1992	0.125
PL14		San Martín, La Pampa, Argentina[a]	Diseased larvae	1995	0.0625
PL15		La Plata, Buenos Aires, Argentina[a]	Diseased larvae	1995	0.0625
PL21		New Zealand[b]	Diseased larvae	1995	0.03125
PL25		New Zealand[b]	Diseased larvae	1995	0.125
PL30		New Zealand[b]	Diseased larvae	1995	0.03125
PL32		Arroyo Alegre, Córdoba, Argentina[a]	Diseased larvae	1995	0.03125
PL33		Cháscomus, Buenos Aires, Argentina[a]	Diseased larvae	1995	0.5
PL34		Valle Medio, Rio Negro, Argentina[a]	Diseased larvae	1995	0.0625
PL35		Chimpay, Rio Negro, Argentina[a]	Diseased larvae	1995	0.125
PL41		Lecce, Italy[c]	Diseased larvae	1991	0.125
PL42		Modena, Italy[c]	Diseased larvae	1992	0.015
PL44		Padova, Italy[c]	Diseased larvae	1995	0.25
PL45		Vanchuse, France[d]	Diseased larvae	1995	0.03125
PL49		Var, France[d]	Diseased larvae	1995	0.0625
PL51		Lincoln, Buenos Aires, Argentina[a]	Diseased larvae	1995	0.5
PL57		Uppsala, Sweden[e]	Diseased larvae	1995	0.125
PL58		Uppsala, Sweden[e]	Diseased larvae	1995	0.03125
PL59		Uppsala, Sweden[e]	Diseased larvae	1995	0.015
PL64		Ranchos, Buenos Aires, Argentina[a]	Honey	1995	0.5
PL71		Grye, Poland[f]	Received as a culture	1996	0.25
PL73		Skierne, Poland[f]	Received as a culture	1996	0.125
PL74	CCM 4483	Czech Republic[g]	Culture	–	0.03125
PL76	CCM 4485	Czech Republic[g]	Culture	–	0.125
PL78		Argentina[a]	Honey	1996	0.125
PL81		Dufaur, Buenos Aires, Argentina[a]	Diseased larvae	1996	0.0625
PL85		New York, USA[h]	Diseased larvae	1996	0.0625
PL89		Holzkirchen, Germany[i]	Diseased larvae	1994	0.0625
PL90		Holzkirchen, Germany[i]	Diseased larvae	1994	0.125
PL91		Holzkirchen, Germany[i]	Diseased larvae	1994	0.03125
PL95		Devon, UK[j]	Received as a culture	1997	0.25
PL96		Leicester, UK[j]	Received as a culture	1997	0.125
PL97		Kent, UK[j]	Received as a culture	1997	0.0625
PL99		Argentina[a]	Commercial honey	1997	0.125
PL100		Tunicia[a]	Honey	1997	0.125
PL101	ATCC 9454	USA[k]	Received as a culture	–	0.125
PL102		Argentina[a]	Commercial honey	1998	0.5
PL103	NRRL B 3555	USA[l]	Received as a culture	–	0.125
PL104	ATCC 2574	USA[k]	Received as a culture	–	0.03125
PL155		Magdalena, Buenos Aires, Argentina[a]	Honey	1999	0.0625
PL202		Italy[a]	Commercial honey	1999	0.5
PL206		Gral. Pinto, Buenos Aires, Argentina[a]	Honey	1999	0.25
PL212		Toronto, Canada[a]	Commercial honey	1999	0.125
PL213		Toronto, Canada[a]	Commercial honey	1999	0.25
PL214		Toronto, Canada[a]	Commercial honey	1999	0.125
PL231		France[a]	Commercial honey	1999	0.0625
PL238		France[a]	Commercial honey	1999	0.25
PL252		Spain[m]	Received as a culture	2000	0.125
PL255		Spain[m]	Received as a culture	2000	0.0625
PL284		Nueva Palmira, Uruguay[n]	Received as a culture	2000	0.03125
PL286		Paysandú, Uruguay[n]	Received as a culture	2000	0.03125

Table 1 (*Continued*)

Strain	Other designation	Geographical origin	Source	Year	MIC
PL287		Nueva Palmira, Uruguay[n]	Received as a culture	2000	0.03125
PL289	P41	Japan[o]	Received as a culture	2000	0.03125
PL290	P55	Japan[o]	Received as a culture	2000	0.03125
PL295		USA[a]	Commercial honey	2000	0.015
PL304		West Flanders, Belgium[p]	Received as a culture	2001	0.125
PL305		Brabant, Belgium[p]	Received as a culture	2001	0.03125
PL316		Mar Chiquita, Buenos Aires, Argentina[a]	Honey	2001	0.125
PL318		Chile[m]	Received as a culture	2001	0.5
PL319		Chile[m]	Received as a culture	2001	0.125
PL339		Chile[m]	Received as a culture	2001	0.125
PL368		Buzios, Brazil[a]	Commercial honey	2001	0.03125
PL373		Boston, USA[a]	Commercial honey	2001	0.015
PL374		Boston, USA[a]	Commercial honey	2001	0.015
PL380		Brandsen, Buenos Aires, Argentina[a]	Honey	2001	0.0039
PL381		La Plata, Buenos Aires, Argentina[a]	Honey	2001	0.03125
Pseudomonas aeruginosa[q]	ATCC 27853		Received as a culture	–	>32
Staphylococcus aureus[q]	ATCC 29213		Received as a culture	–	2
Escherichia coli[q]	ATCC 29922		Received as a culture	–	>32

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[e] I. Fries, Bee Division, Swedish University of Agricultural Sciences, Uppsala, Sweden.
[f] M. Jelinski, Instytut Weterynarii, Swarzdez, Poland.
[g] CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCT, Coleçao de Culturas Tropical, Fundaçao André Tosello, Campinas, SP, Brazil.
[h] Dr. Marta Gilliam, Carl Hayden Honey Bee Research Center, USDA-ARS, Tucson, AZ, USA.
[i] U. Rdest, Biozentrum der Universität Würzburg, Lehrstuhl Mikrobiologie, Germany.
[j] B. Dancer, School of Pure and Applied Biology, University of Wales, Cardiff, UK.
[k] ATCC, American Type Culture Collection, Rockville, MD, USA.
[l] NRRL, Northern Utilization Research and Development Division, Peoria, IL, USA.
[m] P. Avalos, Laboratorio y Estación Cuarentenaria Lo Aguirre, SAG, Chile.
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2.3. Field experiments

The efficiency of tylosin tartrate for the control of AFB on diseased bee colonies was evaluated on three field experiments conducted at the Faculty of Agricultural Science Experimental Field, UNLP, La Plata 35 °S latitude, 57 °W longitude. In all experiments, colonies of honeybees derived from *Apis mellifera ligustica* L. were used. Queens were marked and their wings were clipped to avoid swarming. Colonies were distributed in a completely randomized design. The experimental procedures for inoculation were conducted as described by Alippi et al. (1999).

The amount of healthy brood, adult bees and larvae with AFB-clinical symptoms were measured in the same way in all of the experiments. Healthy brood cells (larvae and pupae) were quantified according to the following scale: grade 0, absence of brood cells; grade 0.5, between 1 and 499 brood cells; grade 1, between 500 and 3499 brood cells; grade 2, between 4000 and 7999 brood cells; grade 3, between 8000 and 11,499 brood cells; grade 4, between 12,000 and 15,999 brood cells and grade 5, between 16,000 and more than 16,000. The number of adult bees were quantified in a similar manner, with a minimum of grade 0 and a maximum grade 5, being grade 0,

absence of adult bees and grade 5, 16,000 or more than 16,000 bees per colony. Measurement of clinical signs of AFB were estimated according to a seven levels scale, where level 0 is non-detectable AFB symptoms; level 1, between 1 and 10 larvae with clinical signs of AFB; level 2, between 11 and 30 larvae with clinical signs of AFB; level 3, between 31 and 99 larvae with clinical signs of AFB; level 4, more than 100 larvae with clinical signs of AFB; level 5, Queen supersedure due to AFB and level 6, colony death, respectively.

The first experiment started in March and ended in October 2000. The 10 colonies used were from packs graded 4 for bees, 3 for brood and 0 for AFB-infection. Two treatments with five repetitions each were applied: five colonies were treated with 1.5 g of tylosin tartrate (Tylan Esanco®) and five colonies were used as AFB-inoculated controls. The total dose (1.5 g per colony) was divided in six parts preparing 70 g candies (55 g powdered sugar + 15 g 55% sugar syrup + 0.25 g active ingredient (a.i. of tylosin tartrate); cherry jelly was used as an attractant to ensure consumption. For controls a mixture of powdered sugar, sugar syrup and cherry jelly was used. The first candy was supplied as preventive 15 days before the inoculations of colonies with AFB-contaminated combs containing 20 ± 5 scales (Alippi et al., 1999). Inoculated colonies were evaluated once a month starting 30 days after inoculation. Each candy was replaced with a new one every 2 weeks. At the end of the experiment disease recurrence was also evaluated.

The second experiment was carried out between April and August 2001. The 10 colonies used were from packs graded 4 for bees, 3 for brood and 0 for AFB disease signs. Inoculations and evaluation of colonies were managed like those in the first experiment, but with modifications in doses and form of administration of the antibiotic. Two treatments with five repetitions each were applied: five colonies were treated with 0.750 g tylosin tartrate in a 70 g candy 15 days before inoculation and five colonies used as AFB-inoculated controls were supplied with a 70 g candy. Four monthly inspections were made starting 30 days after infection.

The third experiment took place between May and October 2002. Twelve colonies were used and were graded 4 for bees, 3 for brood and 0 for AFB-infection. The differences with the former experiment were: the treatments were applied by means of monthly supplied syrup. Six colonies were treated with 0.750 g tylosin tartrate and six colonies were used as AFB-inoculated controls (50% sacarose syrup). The total dose of tylosin for the first group was provided at the first application with 750 cc of syrup 30 days after infection. Four monthly applications of 50% syrup without antibiotic were made afterwards. Controls were supplied in five monthly applications consisting of 750 cc 50% sacarose syrup. Taste was improved by adding 0.5 ml raspberry essence to each jar.

2.4. Statistical analysis

Wilcoxon two-sample test was used for analyzed significant differences ($p < 0.05$) between colonies treated with tylosin and untreated controls in each experiment as described by Spivak and Reuter (2001). Mantel Haenszel Chi-square test and also Fisher exact test (two tails), specially suited for small samples, were run in order to estimate the infection among treatments ($p < 0.05$) in all three experiments. In the same way, the Fisher exact test was used to estimate significant differences in the number of brood cells and that of adult bees between tylosin treatments and AFB-inoculated controls. Results from all three experiments were combined for that purpose (Spivak and Reuter, 2001).

3. Results and discussion

3.1. Minimum inhibitory concentrations of tylosin

MYPGP probed adequate for growth and interpretation of MIC values of *P.l. larvae* and also results for *P. aeruginosa*, *E. coli* and *S. aureus* are within the acceptable limits for quality control strains (NCCLS, 1999). All *P.l. larvae* strains were highly susceptible to tylosin with MIC values ranging from 0.0078 to 0.5 μg/ml depending upon the tested strain (Fig. 1). Results are summarized in Table 1. These values indicate that very low concentrations of tylosin are required to inhibit the growth of *P.l. larvae*. The appearance of oxytetracycline-resistant *P.l. larvae* strains in many countries has given a high priority to the search of alternatives being tylosin highly effective for the control of the Foulbrood pathogen in vitro

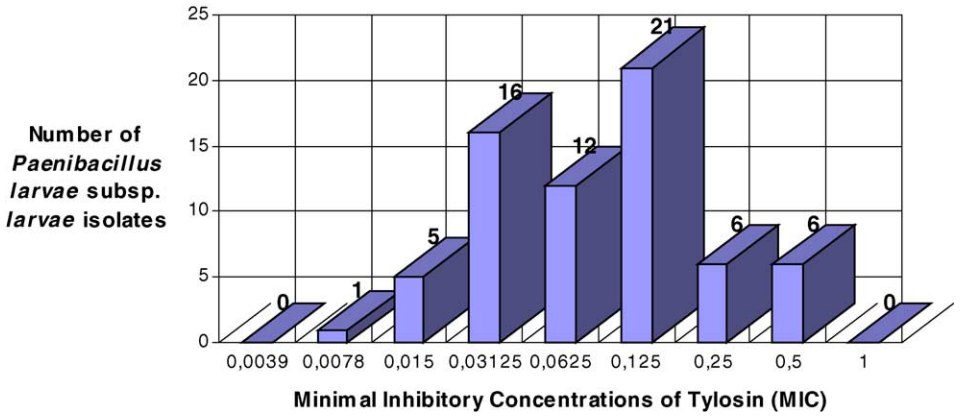

Fig. 1. Frequency of MICs for tylosin between the *Paenibacillus larvae* subsp. *larvae* isolates tested.

when testing 67 strains from different geographical origins. These results are in accordance with previous studies on strains from Japan, where MIC values were between 0.025 and 0.1 μg/ml (Okayama et al., 1996).

The National Committee for Clinical Laboratory Standards do not provide a standard method for determining MIC values for *P.l. larvae* (NCCLS, 1999), nor have breakpoints for antibiotic resistance been established. The values of MIC obtained using MYPGP agar for reference strain of *P. aeruginosa* ATCC 27853 MIC $>$ 32, *S. aureus* ATCC 29213 MIC = 2 and *E. coli* ATCC 29922 MIC $>$ 32 are within the acceptable limits for quality control strains, being $>$32, between 0.5 and 4 μg/ml and $>$32, respectively. Isolates could be considered as susceptible when their MICs were $\leq$8, intermediate for MICs of 16 and resistant for MICs $\geq$ 32, as suggested for tylmicosin (NCCLS, 1999) or resistant when their MICs were $\leq$4 as considered for many antibiotics (Gales et al., 2001). Based upon the results of the present study, there are not any *P.l. larvae* resistant or intermediate strains for tylosin. Further studies that include resistant and/or intermediate *P.l. larvae* strains are needed to corroborate this hypothesis, but until present there is not tylosin resistance reported for this species.

3.2. Field experiments

In the first field experiment, three out of five AFB-inoculated control colonies showed symptoms 60 days after inoculation, and 150 days after inoculation, all colonies showed clinical signs ranging from level 1 to level 6. On the other hand, the colonies treated with tylosin showed no symptoms at all. The results from the first field experiment showed that the disease was controlled in all of the treated colonies and no recurrence of the disease was observed after the end of the experiment by using six candies of 250 mg tylosin (Table 2 and Fig. 2).

During the second field experiment two colonies treated with tylosin showed symptoms 90 days after inoculation (level 1). One colony recovered 120 days after inoculation (level 0) and the other remained with less than three larvae affected (level 1) (Table 3). One of the AFB-inoculated controls showed symptoms after 30 days. After 90 days, all AFB-inoculated controls showed clinical signs of the disease and the infection increased throughout the end of the experiment (ranging from level 1 to level 6) (Table 3). The results from the second field experiment showed that the disease was controlled in 80% of the treated colonies and no recurrence of the disease was observed after the end of the experiment by using one candy of 750 mg of tylosin (Table 3 and Fig. 2).

In the third field experiment, after 90 and 120 days of the infection, in the tylosin treatments, no AFB diseased larvae were found, and after 150 days one colony exhibited two larvae with clinical symptoms of AFB (level 1). The AFB-inoculated controls showed clinical signs in three of them 60 days after infection (level 1). Two of them died after 90 and 120 days, respectively (level 6) (Table 4 and Fig. 2).

Table 2
Clinical symptoms of AFB and amount of healthy brood and adult bees per colony and inspection date during the first field experiment starting in March 29, 2000

Treatment	Colony number	May 29			July 11			August 11			September 11			October 17		
		Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB
Control	118	2	3	0	2	4	1	2	3	2	3	4	4	4	5	4
Control	107	2	3	0	1	2	1	0	0	6	0	0	6	0	0	6
Control	144	2	3	0	2	4	1	2	3	0	2	3	5	2	3	5
Control	71	2	4	0	2	3	0	3	4	0	3	4	0	5	5	1
Control	102	2	3	0	1	3	0	2	3	0	2	3	5	0	0	6
Tylosin	42	2	4	0	2	4	0	2	2	0	3	4	0	4	5	0
Tylosin	111	2	4	0	2	4	0	2	4	0	3	5	0	4	5	0
Tylosin	108	2	4	0	2	4	0	2	3	0	4	5	0	8	5	0
Tylosin	112	2	4	0	2	4	0	2	2	0	3	4	0	4	5	0
Tylosin	41	2	3	0	2	4	0	3	4	0	4	5	0	3	5	0

Overall the results were consistent over 3 years of field experiments, in relation to infection level variable, both the Wilcoxon and the Fisher tests showed similar results. The tylosin treated colonies showed significant differences respect compared to the AFB-inoculated controls ($p < 0.05$). Likewise, the analysis of the infection variable in all three experiments together with the Fisher and Mantel Haenszel Chi-square tests gave significant differences for tylosin compared to those of the AFB-inoculated controls (Fisher $p < 0.05$; Mantel Haenszel Chi-square = 17,364, $p < 0.05$) (Fig. 2). The result of the analysis of the brood variable in all three experiments under the Fisher test showed significant differences for tylosin compared to the AFB-inoculated controls ($p < 0.05$), which confirms the high effectiveness of tylosin. The analysis for the adult bee variable in all three experiments under the Fisher test showed no significant differences between tylosin and the AFB-inoculated controls ($p > 0.05$). The reason for this could lay in the fact that during the autumn–winter season workers show an increase in lifespan associated with the decrease in the queen's egg laying capabilities. Thus, the disease had no noticeable effects on adults throughout the experiments.

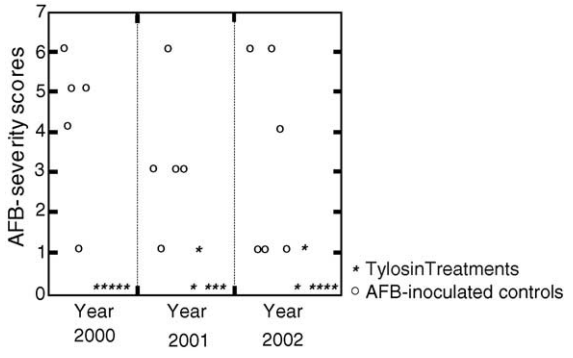

Fig. 2. Severity scores of larvae with AFB-clinical symptoms in tylosin treated and control colonies at the last inspection date during 2000, 2001 and 2002 field experiments.

Our results agree with publication data from other researchers about the effectiveness of tylosin for controlling clinical symptoms of AFB. Our previous studies (Alippi et al., 1999) demonstrated the efficacy of tylosin to control AFB by using a formulation of 1500 mg a.i. of tylosin tartrate applied in extender patties or in paper pack in suppressing AFB clinical signs after 1 year after treatment. Peng et al. (1996) found that dosages of 100 and 200 mg of tylosin protected the colonies for 3 and 4 weeks, respectively, but an additional feeding of tylosin at 100, 200, 400 and 800 mg doses can eliminate the signs of infection for an additional 3-week period. Peng et al. (1996) also noticed that bees were reluctant to accept 800 mg tylosin/7 g sugar dose, taking more than 4 weeks for its consumption. In our study bees took less than 21 days to consume 750 mg tylosin tartrate supplied with syrup, probably due to the addition of raspberry essence to improve the taste. Elzen et al. (2002a,b) working with colonies with different levels of infection with oxytetracycline-resistant AFB from a commercial apiary managed to control the infection in 15 and 45 days, respectively, by sprinkling 200 and 400 mg powdered tylosin tartrate. No re-infection was

Table 3
Clinical symptoms of AFB and amount of healthy brood and adult bees per colony and inspection date during the second field experiment starting in April 13, 2001

Treatment	Colony number	May 13			June 10			July 8			August 5		
		Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB
Control	16 D	1	2	0	1	2	1	2	2	3	1	2	3
Control	10 D	1	3	0	2	3	0	2	3	1	2	3	1
Control	14 D	2	3	3	2	3	4	2	2	5	0	0	6
Control	17 D	2	3	0	2	3	2	2	3	3	2	4	3
Control	4 D	2	3	0	2	3	1	2	3	2	2	4	3
Tylosin	3 E	2	3	0	2	3	0	2	3	0	2	3	0
Tylosin	5 E	2	3	0	1	2	0	2	3	1	2	3	1
Tylosin	15 E	2	3	0	2	3	0	2	3	1	2	3	0
Tylosin	6 E	2	3	0	1	2	0	1	2	0	1	2	0
Tylosin	12 E	2	3	0	2	3	0	2	3	0	2	3	0

observed 7 months after treatment at both doses, while with doses of 100 mg colonies showed clinical signs of the disease after 3 weeks. In another trial, Elzen et al. (2002a,b) demonstrated that a total dose of 600 mg tylosin over 3 weeks applied as a dust in a powered sugar mixture effectively controls AFB during 3 months, the authors also tested a greasy patty method of application with an equivalent weekly tylosin dosage and found that the method was also effective. However, in all colonies treated with patties bee populations were significantly reduced due to the invasion and proliferation of the small hive beetle *Aethina tumida* that consume the food sources of a colony, including a greasy patty.

Regarding the form of application and the doses of tylosin used in our study, no significant differences were found among our three experiments ($p < 0.005$), but taking into account that tylosin is converted into desmycosin that could remain stable in honey over 9 months (Kochanski, 2004a,b) we suggest to use a single dose of 750 mg and also in autumn treatment to prevent persistent residues in honey. Applications in syrup are not convenient because it is stored directly by the bees, and any applications during the nectar flow when honey is being stored should be avoided.

Nevertheless, the results obtained from Kochanski (2004b) were from honeys prepared by adding tylosin under laboratory conditions. Further studies are needed to determine the fate of tylosin and desmycosin residues in honey and royal jelly under field conditions at the recommended doses for field trials, and also the disposition profile of tylosin and desmycosin among honeybees, larvae and pupae for understanding its pharmacokinetics in bee colonies.

Table 4
Clinical symptoms of AFB and amount of healthy brood and adult bees per colony and inspection date during the first field experiment starting in May 22, 2002

Treatment	Colony number	June 22			July 21			August 14			September 18			October 20		
		Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB	Brood	Bees	AFB
Control	4 C	2	3	1	2	3	1	2	2	1	1	2	5	0	0	6
Control	11 C	3	2	0	2	3	0	2	3	0	2	3	0	3	5	1
Control	14 C	2	3	0	1	3	0	2	3	0	2	3	0	3	4	1
Control	3 C	2	3	2	2	3	3	1	2	5	0	0	6	0	0	6
Control	8 C	2	3	1	2	3	2	2	3	1	2	3	3	2	2	4
Control	9 C	2	4	1	2	3	0	2	3	0	2	3	0	4	4	1
Tylosin	41 A	2	3	0	2	3	0	2	3	0	2	2	0	2	3	0
Tylosin	30 A	2	3	0	1	3	0	1	2	0	1	2	0	2	3	1
Tylosin	2 A	2	3	0	1	3	0	2	2	0	1	2	0	2	2	0
Tylosin	5 A	2	3	0	1	3	0	1	3	0	2	3	0	2	3	0
Tylosin	7 A	2	4	0	2	3	0	1	3	0	2	2	0	2	3	0
Tylosin	17 A	2	3	0	2	3	0	2	3	0	2	3	0	3	3	0

Acknowledgments

This research was supported by CIC (Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina). AMA and MRD are Career Investigators of CIC and FJR is a recipient of a scholarship from CONICET, Argentina.

The authors are grateful to S. Dimenna for technical assistance in laboratory and field studies and to Esanco Laboratory, Buenos Aires, Argentina for providing the antibiotic tylosin used for field studies.

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