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Screening alternative antibiotics against oxytetracycline-susceptible and -resistant *Paenibacillus larvae*

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Abstract – Since resistance of the causative organism of American foulbrood, *Paenibacillus larvae* subsp. *larvae*, to oxytetracycline (OTC) is becoming widespread in the United States, we began a search for effective alternative antibiotics. We investigated the sensitivity of *P. l. larvae* to 27 antibiotics, which were primarily ones already registered with the US Food and Drug Administration for agricultural uses. Bacterial resistance to OTC also conferred resistance to other tetracyclines, although the level of resistance varied. The most active antibiotics screened that are currently used in agriculture were erythromycin, lincomycin, monensin, and tylosin. Rifampicin was by far the most active antibiotic tested, but since it is used against tuberculosis, registration of this material for agricultural use is unlikely.

***Apis mellifera* / *Paenibacillus larvae larvae* / antibiotic / resistance / American foulbrood**

1. INTRODUCTION

American foulbrood (AFB) and European foulbrood (EFB) are two bacterial diseases of honey bee brood, caused by *Paenibacillus larvae* subsp. *larvae* (formerly *Bacillus larvae*) and *Melissococcus pluton*, respectively. AFB has traditionally been controlled by burning infected colonies, and this practice is still required in some areas. In order to lessen this financial loss to beekeepers, pharmacological methods for foulbrood prevention and control were developed.

The first medications tested were synthetic antibacterials: the sulfa drugs,

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particularly sulfathiazole (Haseman and Childers, 1944; Eckert, 1947; Reinhardt, 1947; Johnson, 1948; Katznelson and Gooderham, 1949; Katznelson, 1950). They were effective against AFB, but their stability and consequent residues in honey caused problems, and the registration was allowed to lapse in the 1970s (Shimanuki and Knox, 1994).

Tests on antibiotics began in the late 1940s as well (cited by Katznelson, 1950). These early studies showed that Aureomycin[1] (chlortetracycline) was active, while penicillin, chloramphenicol, streptomycin, and others were considerably less active against *P. l. larvae*. Oxytetracycline (OTC, Terramycin[1]), usually as its hydrochloride, has been used since the early 1950s for the prevention and control of AFB and EFB (Gochnauer, 1951; Katznelson et al., 1952). It remains after many years the only approved drug treatment for the foulbrood diseases in the United States. Recently, however, strains of *P. l. larvae* showing resistance to OTC have been discovered in Argentina (Alippi, 2000) as well as in multiple areas in the United States (Miyagi et al., 2000), and there is now general concern about widespread resistance.

Other antibiotics have been investigated as treatments for AFB. Tylosin (a macrolide antibiotic) was first investigated for control of AFB about thirty years ago (Hitchcock et al., 1970; Moffett et al., 1970; Peng et al., 1996) and was found to be much more stable in sugar syrup than was OTC (Kochansky et al., 1999). Erythromycin (another macrolide) was first tested in 1955 (Katznelson et al., 1955; Katznelson, 1956; see also Hitchcock, 1964), but reports on its efficacy differed. Katznelson et al. (1955) and Moffett et al. (1958) found it to be ineffective against AFB, but Machova (1970) found good efficacy against AFB, and it has been reported to be effective against EFB (Wilson and Moffet, 1957; Wilson, 1962). Machova (1970) also tested other antibiotics in a study of antibiotic sensitivity of ten strains of *P. l. larvae* isolated from various regions of Czechoslovakia. In addition to the activity of erythromycin cited above, good sensitivity was obtained to bacitracin and the tetracyclines, with lower sensitivity to 10 others.

A large number of *P. l. larvae* strains in Japan were tested for antibiotic sensitivity (Okayama et al., 1996). Mirosamycin, another macrolide antibiotic, has been studied as a result of its high activity in this initial screen (Nakajima et al., 1998). Ampicillin was another antibiotic with high activity in vitro, but when tested in beehives it gave high residues in honey but only very low levels in larvae, casting doubt on its utility in disease control (Nakajima et al., 1997).

In view of the increasing incidence of OTC-resistant *P. l. larvae* in the US, we embarked on a screening program to identify additional antibiotics that might be useful in the prevention and treatment of AFB. We now report the results of laboratory tests evaluating a variety of antibiotics for ability to inhibit both OTC-resistant and OTC-susceptible strains of *P. l. larvae*.

2. MATERIALS AND METHODS

2.1. Antibiotics

Antibiotics (Tab. I) were purchased from Sigma or Fluka and a stock solution (200 mg/L in 50% methanol) was prepared for each. Successive dilutions were prepared to yield 60, 20, 6, 2, 0.6, and 0.2 mg/L (rifampicin was also tested at 0.06 mg/L). Antibiotic test disks (6.35 mm, No. 740-E,

[1] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Table I. Classes of antibiotics tested vs. American foulbrood.

Tetracyclines	Aminocyclitols	Macrolides
chlortetracycline	apramycin	erythromycin
demeclocycline*	spectinomycin	oleandomycin
doxycycline		tylosin
minocycline*	Chloramphenicols	
oxytetracycline	chloramphenicol	Lincosaminides
tetracycline		clindamycin
	Peptides	lincomycin
Aminoglycosides	bacitracin	
amikacin	colistin	Polyether ionophores
gentamycin	polymixin	monensin
kanamycin		
neomycin	Glycopeptides	Ansamacrolides
paromomycin*	vancomycin*	rifampicin*
streptomycin		
tobramycin*		

* Not FDA registered for any agricultural uses as of December 2000.

Schleicher and Schuell), which had been shown gravimetrically to absorb 0.02 g = 20 μL of water, were dipped in the antibiotic solution to saturation and allowed to dry. This resulted in a series of disks containing 4, 1.2, 0.4, 0.12, 0.04, 0.012, and 0.004 μg of each antibiotic/disk.

2.2. Bioassay

Antibiotics were tested against *P. l. larvae* by our standard disk diffusion method (Shimanuki and Knox, 1991), similar to the method reported by Feldlaufer et al. (1993). The susceptible strain of *P. l. larvae* was obtained from New Jersey and an OTC-resistant strain was obtained from Minnesota. A stock spore suspension (approximately 2×10^8 spores/mL) of each strain was prepared by mixing 3–5 scales (the dried remains of diseased honey bee larvae containing the bacterial spores) with sterile water (9 mL) in a screw-capped tube. Before each use, the suspension was heat-shocked at 80 °C for 10 minutes to kill any non-sporeforming bacteria. For the bioassay, 0.2 mL of the stock suspension was spread over the surface of freshly-prepared brain-heart infusion agar (BHI) plates (brain-heart infusion, Difco Laboratories, Detroit, MI, fortified with thiamine hydrochloride (0.1 mg/L), 2% agar, and adjusted to pH 6.6 with hydrochloric acid). The antibiotic-treated disks were positioned in the center of the BHI plates and the plates were incubated at 34 °C in the dark. The diameters of the zones of inhibition were measured after 72 hours. We did not make any correction for disk diameter. If there was a visible zone of inhibition around the disk, the total diameter of that zone was recorded and the compound was termed 'active' at that concentration; if no such zone was visible, the diameter was recorded as zero and the compound was therefore 'inactive'. Negative controls consisted of disks treated with 50% methanol, and commercial sensitivity test disks (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) treated with 5 μg of tetracycline were used as positive controls.

The tests against tetracyclines in Table II and in the general survey in Table III were run only once, since only an indication of

the most active antibiotics was desired. The tests of selected antibiotics against resistant and susceptible AFB reported in Table IV were replicated 3–4 (mostly 4) times and are reported as mean ± standard error. Data reduction and plotting were performed with GraphPad Prism ver. 3.0 (GraphPad Software, Inc. San Diego, CA).

Table II. Tetracycline antibiotics tested against resistant and susceptible *P. l. larvae.*

Antibiotic	Strain[1]	4 μg/disc	1.2	0.4	0.12	0.04	0.012
Oxytetracycline	S	50	42	35	20	0	0
	R	12	0	0	0	0	0
Chlortetracycline	S	58	50	ND	45	22	0
	R	22	18	ND	10	0	0
Demeclocycline	S	75	70	45	35	25	10
	R	15	15	15	12	0	0
Doxycycline	S	63	58	52	50	40	0
	R	33	20	15	14	10	0
Minocycline	S	58	70	40	ND	16	0
	R	70	68	33	ND	20	0
Tetracycline	S	57	50	54	28	25	0
	R	14	15	10	0	0	0

[1] S = susceptible (New Jersey), R = resistant (Minnesota).

Table III. Initial screens of antibiotics vs. susceptible *P. l. larvae.*

Antibiotic	4 μg/disc	1.2	0.4	0.12	0.04	0.012	0.004
Oxytetracycline	50	42	28	20	16		
Tylosin	40	34	28	17	12	0	
Erythromycin	57	50	35	30	15	0	
Oleandomycin	50	20	0	0	0	0	
Clindamycin	75	55	20	0	0	0	
Lincomycin	70	70	57	47	25		
Bacitracin	36	25	13	0	0	0	
Chloramphenicol	40	25	0	0	0	0	
Gentamycin	12	0	0	0	0	0	
Monensin	50	36	40	32	15	0	
Rifampicin	65	60	52	48	40	30	28
Tobramycin	10	0	12	0	0	0	
Vancomycin	28	15	20	0	0	0	

Numbers in table are diameters of inhibition zones around the treated disks at the various concentrations, tested against susceptible (New Jersey) *P. l. larvae.*
Amikacin, apramycin, colistin, kanamycin, neomycin, paromomycin, polymixin, spectinomycin, and streptomycin were inactive at all concentrations tested.

Table IV. Comparison of activity of selected antibiotics against susceptible and resistant *P. l. larvae*.

Antibiotic	4 µg/disk	1.2 µg/disk	0.4 µg/disk	0.12 µg/disk	0.06 µg/disk	0.012 µg/disk	0.006 µg/disk	0.0012 µg/disk
OTC (S)	46.3 ± 2.8	40.5 ± 2.4	30.8 ± 3.4	16.8 ± 5.9	7.3 ± 4.4	0.0		
OTC (R)	6.3 ± 3.6	2.8 ± 2.8	0.0	0.0	0.0	0.0		
Erythromycin (S)	52.0 ± 1.2	45.0 ± 1.2	34.8 ± 0.5	25.0 ± 1.8	7.8 ± 4.5	0.0		
Erythromycin (R)	53.3 ± 1.2	44.5 ± 0.5	34.5 ± 0.9	25.5 ± 1.3	14.3 ± 1.4	2.0 ± 2.0		
Lincomycin (S)	39.3 ± 3.0	28.3 ± 1.2	18.0 ± 1.5	5.3 ± 3.1	2.8 ± 2.8	0.0		
Lincomycin (R)	39.8 ± 1.0	30.8 ± 0.5	18.5 ± 1.2	13.7 ± 0.9	5.8 ± 3.3	0.0		
Monensin (S)	33.8 ± 3.2	33.3 ± 2.0	22.3 ± 2.3	7.8 ± 4.5	0.0	0.0		
Monensin (R)	38.8 ± 2.4	36.0 ± 2.3	22.8 ± 3.4	11.3 ± 3.9	0.0	0.0		
Rifampicin (S)	64.8 ± 1.2	58.8 ± 2.4	53.8 ± 0.8	47.0 ± 1.1	35.5 ± 0.9	28.0 ± 0.7	19.3 ± 0.7	14.0 ± 1.0
Rifampicin (R)	66.3 ± 1.3	58.8 ± 1.4	54.3 ± 2.2	46.0 ± 0.7	36.5 ± 0.9	30.0 ± 0.8	20.0 ± 0.0	16.0 ± 1.0
Tylosin (S)	47.5 ± 4.9	36.3 ± 0.5	24.3 ± 1.7	16.5 ± 1.7	2.5 ± 2.5	0.0		
Tylosin (R)	44.5 ± 1.0	36.0 ± 0.7	28.5 ± 0.5	16.8 ± 1.2	5.5 ± 3.2	0.0		

Numbers are diameters of zones of inhibition (mean ± SEM), usually 4 (occasionally 3) replicates.
(S) = susceptible (New Jersey) strain, (R) = resistant (Minnesota) strain.

3. RESULTS AND DISCUSSION

Since OTC is a member of the extended tetracycline class of antibiotics, we tested other members of the class against susceptible and resistant *P. l. larvae*, with results shown in Table II. While some tetracyclines were more effective than others against resistant *P. l. larvae*, in all but one case tetracycline antibiotics were less effective against resistant than against susceptible AFB organisms, indicating considerable cross-resistance. Doxycycline was still moderately active against resistant *P. l. larvae*, but only minocycline showed no apparent loss of activity against the resistant strain. It is not unlikely that this cross-resistance would increase rapidly under selection conditions, and since minocycline has no approved agricultural uses in the US, we did not investigate tetracyclines further.

Twenty-one additional antibiotics (summarized in Tab. I) were screened against susceptible *P. l. larvae* using the same dosage series. The results are shown in Table III. These antibiotics represented 8 classes. Aminoglycoside antibiotics were either completely inactive at all concentrations tested (amikacin, kanamycin, neomycin, paromomycin, or streptomycin) or active only at 4 μg/disk (gentamicin) or 0.4 μg/disk (tobramycin), and were not tested further. Aminocyclitol antibiotics are similar to aminoglycosides structurally, but lack sugar groups bound to the rings. In this class apramycin and spectinomycin were investigated and both were found to be inactive at all concentrations tested. Chloramphenicol, in its own class, was only active down to 1.2 μg/disk and was not tested further.

Of the three peptide antibiotics screened, bacitracin was active down to 0.4 μg/disk, while polymixin and colistin were inactive; none was tested further. Vancomycin, a glycopeptide antibiotic widely used in human medicine against organisms resistant to other antibiotics, was only active down to 0.4 μg/disk.

The remaining antibiotics fell into four categories. The macrolides were represented by tylosin, erythromycin, and oleandomycin, the lincosaminides by lincomycin and clindamycin, the polyether ionophores by monensin, and the ansamacrolides by rifampicin. Oleandomycin and clindamycin were only active down to 1.2 and 0.4 μg/disk, respectively, and were not tested further, but all others were highly active (down to 0.04 μg/disk or below).

The five most active antibiotics from these preliminary screens with the susceptible strain of *P. l. larvae* were tested along with OTC against susceptible and resistant strains of *P. l. larvae* (Tab. IV). As expected, OTC performed very poorly against resistant bacteria. Monensin was the least active of the five, down to 0.12 μg/disk. Erythromycin, tylosin, and lincomycin were active down to 0.04 μg/disk. Okayama et al. (1996) also found tylosin, er thromycin, and lincomycin to be active, with generally high activity from other macrolides, including oleandomycin, which we found to have poor activity. Okayama et al. found good activity from the three tetracyclines they tested (OTC, chlortetracycline, and doxycycline), which suggests that none of their *P. l. larvae* strains was OTC-resistant. As before, rifampicin was outstanding, with activity still present at 0.0012 μg/disk, the lowest dose tested. For the initial screen, extrapolation of the log (concentration) vs. inhibition zone diameter line suggested a minimum inhibitory concentration for rifampicin of only 1.8 μg/L (36 pg/disk). In all cases, these five selected antibiotics were equally active against resistant and susceptible foulbrood. With the exception of rifampicin, all are listed in the FDA "Green Book" (FDA-CVM, 1999) of drugs approved for veterinary use.

Field tests are currently under way using tylosin and lincomycin in colonies against AFB. Preliminary results (to be further described elsewhere) suggest that lincomycin and tylosin are effective against resistant

AFB strains in the field in both Florida and New Jersey, while we have not yet tested erythromycin in the field.

Résumé – Criblage d'antibiotiques alternatifs contre *Paenibacillus larvae* résistant et sensible à l'oxytetracycline. Puisque *Paenibacillus larvae* subsp. *larvae* (anciennement *Bacillus larvae*), l'agent de la loque américaine, est devenu résistant à l'oxytetracycline (OTC) sur une grande échelle aux États-Unis, nous avons recherché des antibiotiques alternatifs qui pourraient efficacement remplacer l'OTC. Nous avons étudié principalement les antibiotiques déjà autorisés par la Food and Drug Administration pour des usages agricoles.
La méthode de diffusion sur disques a été utilisée pour faire un premier criblage d'antibiotiques à des concentrations successives décroissantes à partir de 200 mg/L (4 µg/disque) : 4, 1,2, 0,4, 0,12, 0,04 et 0,12 µg/disque. Seules les substances qui étaient actives à une concentration au moins égale à 6 mg/L (0,12 mg/disque) ont été testées par la suite. Dans la plupart des cas la résistance à l'OTC conférait, avec un niveau variable, une résistance aux autres tetracyclines. Seule la minocycline était active contre les souches de *P. l. larvae* résistantes et les souches sensibles. Les antibiotiques testés les plus actifs (ceux actifs aux doses ≤ 0,04 µg/disque) couramment utilisés en agriculture étaient : l'érythromycine, la lincomycine, la monensine et la tylosine. La rifampicine, bien que non utilisée en agriculture, était de loin l'antibiotique le plus actif, avec une activité étant encore présente à 0,0012 µg/disque mais, étant utilisée contre la tuberculose, il est peu vraisemblable qu'elle reçoive une autorisation pour un usage agricole. La lyncomicine, la tylosine et l'érythromycine étaient actives jusqu'à 0,04 µg/disque et la monensine était la moins active avec une concentration minimum d'inhibition de 0,12 µg/disque.

Apis mellifera* / loque américaine / antibiotique / résistance / *Paenibacillus larvae larvae

Zusammenfassung – Prüfung von alternativen Antibiotika gegen Oxytetrazyklinempfindliche und -resistente Stämme von *Paenibacillus larvae larvae*. Da sich oxytetrazyklinresistente Stämme der Amerikanischen Faulbrut in den USA verbreiten, begannen wir mit der Suche nach alternativen Antibiotika als wirksamen Ersatz. Wir prüften vor allem Antibiotika, die bereits für eine Nutzung im landwirtschaftlichen Bereich zugelassen sind. In Vorversuchen wurden Konzentrationen von Antibiotika mit einer Diffusionsmethode unter Verwendung von Antibiotika Testblättchen erstellt, die von 200 mg/L (4 mg/Testblättchen) ausgehend in einer Reihe von Verdünnungsstufen von 4, 1,2, 0,4, 0,12, und 0,012 mg/disk weitergeführt wurden. Nur Substanzen, die bei einer Konzentration von 6 mg/L (0,12 mg/disk) und geringer wirksam waren, wurden weiterhin getestet. Fast immer war die OTC Resistenz mit Resistenzen zu anderen Tetrazyklinen verbunden, der Grad der Resistenz variierte, nur Minozyclin erwies sich als gleich wirksam gegen resistente und empfindliche Stämme von *P. l. larvae*. Die wirksamsten und zur Zeit in der Landwirtschaft genutzten Antibiotika (wirksam bei 0,04 mg/disk) waren Erythromyzin, Lincomyzin, Monensin und Tylosin. Rifampicin, das nicht in der Landwirtschaft genutzt wird, war das bei weitem aktivste Antibiotikum im Test. Es war noch in einer Konzentration von 0,0012 mg/disk wirksam. Lincomyzin, Tylosin und Monensin waren weniger wirksam als die anderen 4 (geringste Konzentration für eine Hemmung war 0,12 mg/Scheibe).

Antibiotika / Resistenz / Amerikanische Faulbrut / *Paenibacillus larvae larvae*

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