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Enterobacteriaceae Isolated from Honey Bees, *Apis mellifera*, Treated with 2,4-D and Antibiotics[1,2]

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Klebsiella pneumoniae, Shigella sp., *Enterobacter cloacae, Escherichia coli, Enterobacter hafniae, Arizona* sp., *Enterobacter aerogenes, Serratia liquefaciens,* and *Citrobacter* sp. were isolated from the intestinal contents of honey bees, *Apis mellifera,* which were obtained either from untreated colonies, from colonies fed the herbicide 2,4-D, or from colonies fed a combination of oxytetracycline and fumagillin. Antibiotics depressed the growth of Enterobacteriaceae; 2,4-D had little effect on the enteric microflora of bees.

INTRODUCTION

Recently we reported the isolation of Enterobacteriaceae from the intestinal contents of healthy foraging worker honey bees, *Apis mellifera* (Gilliam and Valentine, 1973). *Enterobacter cloacae, Escherichia coli, Enterobacter aerogenes, Shigella,* and *Klebsiella pneumoniae* were identified. No Pseudomonadaceae or *Erwinia* organisms were detected.

Morton et al. (1972) found that 2,4-D was relatively nontoxic when fed in sugar solution to newly emerged honey bees. However, Morton and Moffett (1972) reported that eggs from colonies consuming high levels of 2,4-D did not hatch and that larvae died when they were transferred into colonies consuming this herbicide. Once the herbicide was removed from the honey bee colony, brood development resumed.

Most of the adverse effects of herbicides on honey bees have been attributed to loss of food when plants on which honey bees forage for nectar and pollen are killed by herbicides (Hocking, 1950). Since 2,4-D has a variety of effects on plants, animals, and microorganisms (Barrier et al., 1970; Parry, 1973), we wished to examine Enterobacteriaceae in honey bees fed 2,4-D. Also, we included bees from colonies fed a combination of Terramycin® (oxytetracycline) and Fumidil B® (fumagillin) in our experiments to assess the effects of these antibiotics on the enteric microflora of bees. Terramycin is used to control American foulbrood disease and European foulbrood disease of honey bees, and fumagillin is used to control nosema disease. Antibiotics have been reported to decrease the bacterial flora of bees and increase the yeast flora (Hajsig, 1959; Mitroiu et al., 1966; Tomašec, 1957).

MATERIALS AND METHODS

Colonies were established by placing 3 lb of adult worker honey bees and a laying queen on frames of beeswax foundation. Before bees were fed experimental diets, adult workers were removed from each colony and their intestines were sampled for Enterobacteriaceae. As soon as the bees were placed in the hive, they were fed either the dimethylamine salt of 2,4-D, a

[1] In cooperation with the Arizona Agricultural Experiment Station.

[2] Mention of a proprietary product or company name does not constitute an endorsement of this product by the USDA.

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combination of oxytetracycline and fumagillin, or untreated pollen patties (maintenance diet).

The maintenance diet given to all colonies consisted of 11 lb of pollen, 10 lb of sucrose, 9 lb of Drivert® (92% finely pulverized sucrose +8% invert sugar) (California and Hawaiian Sugar Company), 0.5 lb of Alphacel® (Nutritional Biochemicals Corp.), and 1000 ml of water. This mixture was divided into 1-lb patties which were placed on the top of the frames and replenished weekly. The herbicide 2,4-D was fed at a concentration of 1000 ppm active ingredient by weight in 60% sucrose-water solution from a 1-qt jar placed directly above the frames containing brood (Morton and Moffett, 1972). The mixture of herbicide and syrup was replenished twice a week. Colonies fed antibiotics were given the maintenance diet plus 15 g TM-25® (25 g oxytetracycline/lb of formulation) and 15 g Fumidil B (fumagillin) in addition to 60% sucrose–water solution. The patties consisting of the maintenance diet plus the antibiotics were replenished weekly. Control colonies received the patties and 60% sucrose–water solution. Fresh water was constantly available in 5-qt plastic pails.

Hives were placed outdoors in 12 × 12 × 9 ft Saran® mesh cages. In the first series of experiments, we placed each of the three hives in a separate cage. In the second series of tests, three colonies receiving the same treatment were placed in the same cage. Altogether we examined bees from 12 colonies; 4 control colonies, 4 colonies treated with 2,4-D, and 4 colonies fed antibiotics.

In the first series of tests, three adult worker bees from each colony were examined weekly. The intestinal tracts were aseptically removed and individually homogenized in 2.5 ml sterile 0.85% NaCl as previously described (Gilliam and Prest, 1972). A loopful of the homogenate from each bee was streaked in duplicate on MacConkey agar (Difco) and trypticase soy agar (BBL) in petri dishes. All plates were incubated under aerobic conditions at 37°C for 1 week.

In the second series of tests, every 3 weeks the intestines of two bees from each colony were individually homogenized in 2.5 ml of sterile thioglycollate 135C (BBL). Each homogenate was streaked in duplicate on trypticase soy agar, eugonagar (BBL), and MacConkey agar. One plate was incubated at 25° and one at 37°C under aerobic conditions for 1 week. All cultures were maintained on slants of trypticase soy agar.

All bacterial colonies isolated were stained by the gram reaction. Gram-negative rods were tested for nitrate reduction, oxidase production, and fermentation of glucose (Edwards and Ewing, 1962). All nitrate-positive, oxidase-negative, glucose-positive, gram-negative rods were identified by the API system using 20 biochemical determinations (Analytab Products, Inc., New York) for the identification of Enterobacteriaceae.

The first series of bees were sampled from July, 1971 to January, 1972, when the herbicide-treated and antibiotic-fed colonies died. Bees in the second series of experiments were sampled from September, 1971 to August, 1972 though all herbicide-treated colonies had died by April, 1972, one of the antibiotic-fed colonies died in June, 1972, and a control colony died in May, 1972.

Results

All bees sampled prior to feeding contained members of the Enterobacteriaceae although these bacteria were not identified. Forty-four members of the Enterobacteriaceae representing 9 species were isolated and identified from the 388 bees which we examined. Isolations were made on all media used and at both 25° and 37°C incubation temperatures. Table 1 shows the organisms isolated and their occurrence. *Klebsiella pneumoniae* and *Shigella* sp. were found most frequently.

Table 2 shows the organisms isolated in the first series of tests. Nine isolates were

TABLE 1

SUMMARY OF ENTEROBACTERIACEAE ISOLATED FROM CONTROL BEES, BEES TREATED WITH 2,4-D, AND BEES FED OXYTETRACYCLINE AND FUMAGILLIN

Organism	Number of isolations
Klebsiella pneumoniae	16
Shigella sp.	11
Enterobacter cloacae	5
Escherichia coli	4
Enterobacter hafniae	3
Arizona sp.	2
Enterobacter aerogenes	1
Serratia liquefaciens	1
Citrobacter sp.	1

identified from bees treated with 2,4-D, 6 from bees treated with antibiotics, and only 4 from control bees. No trend was evident except that more bees treated with 2,4-D contained more bacteria belonging to the Enterobacteriaceae in their intestines. All organisms, including members of the Enterobacteriaceae, were eliminated from the intestinal microflora of bees treated with antibiotics 3 months after antibiotics were incorporated in the diets.

TABLE 2

ENTEROBACTERIACEAE ISOLATED FROM HONEY BEES: 1ST SERIES OF TESTS

Organism	Treatment[a]	Date isolated
Enterobacter cloacae	H	7/26/71
Escherichia coli	H	8/2/71
Escherichia coli	H	8/2/71
Escherichia coli	H	8/2/71
Enterobacter cloacae	H	8/16/71
Enterobacter aerogens	H	8/16/71
Klebsiella pneumoniae	A	9/7/71
Enterobacter hafniae	A	9/7/71
Shigella sp.	A	9/13/71
Shigella sp.	A	9/13/71
Escherichia coli	A	9/13/71
Enterobacter hafniae	C	9/20/71
Shigella sp.	A	9/27/71
Shigella sp.	C	10/12/71
Shigella sp.	C	10/12/71
Shigella sp.	H	10/18/71
Shigella sp.	H	10/18/71
Shigella sp.	H	11/8/71
Enterobacter cloacae	C	12/27/71

[a] C = control; H = 2,4-D; A = antibiotics.

Tables 3 gives the results of isolations in the second series of experiments. Nine isolates were from bees treated with 2,4-D, 6 from bees treated with antibiotics, and 10 from control bees. In these tests, Enterobacteriaceae were eliminated from the microflora 1 week after the antibiotics were fed to the bees. However, Enterobacteriaceae again appeared in bees treated with antibiotics after the antibiotics had been fed for 9 months. *Klebsiella pneumoniae* was the most frequent isolate in this series of experiments while *Shigella* sp. occurred most frequently in the bees sampled in the first series of tests.

Citrobacter sp., *Arizona* sp., and *Serratia liquefaciens* were isolated in the second tests but not in the first. *Escherichia coli* and *Enterobacter aerogenes* were isolated in the first tests but not in the second.

TABLE 3

ENTEROBACTERIACEAE ISOLATED FROM HONEY BEES: 2ND SERIES OF TESTS

Organism	Treatments[a]	Date isolated
Klebsiella pneumoniae	C	10/18/71
K. pneumoniae	C	11/9/71
K. pneumoniae	C	11/9/71
K. pneumoniae	C	11/9/71
Arizona sp.	C	11/9/71
Arizona sp.	C	11/9/71
Shigella sp.	C	11/9/71
K. pneumoniae	H	11/9/71
K. pneumoniae	H	11/9/71
K. pneumoniae	H	11/9/71
K. pneumoniae	H	11/9/71
K. pneumoniae	H	11/9/71
K. pneumoniae	H	11/9/71
Serratia liquefaciens	H	11/9/71
Enterobacter cloacae	H	11/9/71
E. cloacae	H	11/9/71
Enterobacter hafniae	C	6/6/72
Shigella sp.	C	6/6/72
Shigella sp.	C	6/6/72
K. pneumoniae	A	6/6/72
K. pneumoniae	A	6/6/72
K. pneumoniae	A	6/6/72
K. pneumoniae	A	6/28/72
K. pneumoniae	A	6/28/72
Citrobacter sp.	A	8/8/72

[a] C = control; H = 2,4-D; A = antibiotics.

Discussion

From the results of this study, bacteria belonging to the Enterobacteriaceae do not appear to be prevalent in the intestines of honey bees in Arizona. However, we have isolated more organisms from free-flying bees (Gilliam and Valentine, 1973). Caging hives therefore may decrease the occurrence of Enterobacteriaceae in bee intestines since Tysset et al. (1969) believed that these organisms are found in nectars or on pollens and contaminate bees during foraging. No members of the Pseudomonadaceae or *Erwinia* were detected in either study.

Most of the isolates were obtained in the fall (August-November), yet no trends were evident except that bees from colonies treated with 2,4-D contained a few more members of the Enterobacteriaceae in their intestines.

Feeding 2,4-D to colonies of honey bees affects longevity of the bees. Colonies fed 2,4-D survived only 6 months in the first test and 7 months in the second test. We attribute the death of these colonies to the ovicidal and larvicidal effects of the herbicide (Morton and Moffett, 1972).

Organisms isolated in the present study which were not reported in our previous paper (Gilliam and Valentine, 1973) included *Enterobacter hafniae, Arizona* sp., *Serratia liquefaciens,* and *Citrobacter* sp.

Obviously, none of the Enterobacteriaceae isolated are symbiotes in the intestines of bees since many bees contained no Enterobacteriaceae in their guts. Our results would seem to agree with those of Tysset et al. (1969), which indicated that these organisms are in transit in the intestine of the bee. However, unlike Tysset et al., we found no evidence that enteric bacteria could adapt to the intestine of the bee by producing neotypes which could be differentiated by minor biochemical characteristics.

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