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DEPARTMENT AND CLINIC OF INFECTIOUS DISEASES OF ANIMALS,
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Enterococcus septicemia of *Apis mellifera* L.

Posocznica *Apis mellifera* spowodowana zakażeniem enterokokami

Septicemic diseases are the most common bacterial infections in insects (7). This pathological state is associated with the presence of bacteria and/or their toxins in the insect body cavity. Pathological conditions result from destruction of insect body tissues by an invader (20). The proof of septicemia in insects is by positive hemolymph and tissues, mainly muscles, culture and smears. As a rule septicemia appears in insects under stress as a secondary infection. Parasitic invasions which are strong stressogenic factors are often followed by bacterial septicemia.

Bacteria associated with the bee are widely distributed in soil, water, air, stored food, surface of plants and on skin of other living organisms (9, 10, 11, 12, 15). *E. faecalis*, like other species of the genus *Enterococcus*, occurs widely in the environment, particularly in feces of vertebrates (18), on many plants and their flowers, in soils (21), and on insects (7). Gram-positive catalase-positive cocci have also been isolated from apiarian sources from healthy honey bees (9) and from European foulbrood sick bee larvae, as a common secondary invader in the disease (1, 24).

Streptococci are rarely mutualistic or comensals in insects but rather are transient residents that enter the digestive tract during feeding or are transferred mechanically (15). Gram-positive streptococci found in bees are temporary residents of higher plants, particularly of the floral organs, and are in transit in the digestive tract of the healthy bee that becomes infected while collecting nectar and pollen (23). Catalase-negative cocci of the *Enterococcus* group (*S. faecalis*, *S. faecium*, *S. durans*, *S. equinus* and *S. avium*) were predominant in frass of feral bee colonies. Catalase-negative cocci were also isolated from the wax moth culture. *E. faecalis* is brought in honeybee colonies by bees from the field and it becomes temporarily established as a common secondary invader in the disease where European foulbrood breaks out (24).

Under stress conditions such as elevated temperature and high relative humidity, wounding, *Enterococcus faecalis* (*Streptococcus faecalis*) has been reported to cause mortality in several species of insects (4, 5). Most infected insects die within a few days after penetration of bacterial cells into the body cavity. Affected insects are weak, unable to fly and have diarrhea. Moribund insects had *E. faecalis* in the blood and in the gut. Cultures of the organism isolated from insects that died after natural infections produced more mortality after injection into the body cavity of apparently healthy insects.

This paper described septicemic conditons produced in the honeybee by natural and experimental infectin of *E. faecalis*, cultural and biochemical characteristics and antibiotic resistance pattern of the *Enterococcus* involved.

MATERIAL AND METHODS

A case history. Heavy losses have been noted in bees in 9 colonies in one beeyard situated in the Southern Finland, 60-70 km west of Helsinki in spring 1995. Six colonies died in March-April and only one survived at May. Colonies died in March-April. All dead hives had plenty of food left and they had even brood. The main symptom in every colony was weakness and heavy diarrhea. Brood was free from infectious diseases (American foulbrood, European foulbrood, sacbrood).

Varroa jacobsoni was found 2-3 years ago and colonies have been treated since that time with Bayvarol and Apistan. Irrespective of the treatment heavy infestation persisted in one colony. The reinfection of *Varroa* is possible because the nearest neighbouring beeyard lies a distance of 0.5 km and within the radius of 1.5 km there are several beeyards.

Isolation and identification of *Enterococcus faecalis*. As specimens for isolation of microorganisms served dead bees and samples of bee faces. The alimentary tract and thoracic muscles obtained under sterile conditions were homogenized in distilled water and an aliquot of 0.1 ml of homogenate was inoculated directly onto duplicate plates of agar of nutrient broth (Oxoid No 2) and malt extract-yeast extract agar containing 1% glucose (25). Feces after dilution in a small volume of physiological salt saline were streaked on the above media. The samples of the same specimen were also streaked on Sabouraud dextrose agar. One set of plates was incubated under aerobic conditions at 35°C for 48 hr and the other at 25°C for 10 days.

The selected microbial colonies were restreaked on the media used for initial isolation in order to test for and prepare pure cultures. Before biochemical testing, the isolated microorganisms were maintained in slants of nutrient agar of Sabouraud dextrose agar at refrigerator.

Cocci were tested and identified using the API-Strept. system according to the manufacturer's directions. In the tests characteristics differentiating the species of *Enterococcus* were included (18).

Bacterial isolates were stained by the Gram reaction. Gram-negative rods were tested for nitrite reduction, oxidase production and fermentation of glucose (8). Gram-negative, glucose positive and nitrite and oxidase negative rods were identified by the API-20E system using twenty biochemical determinations for the identification of members of the *Enterobacteriaceae*.

Morphological and biochemical properties of yeasts were checked according to Wickerham (25).

Parasitological examinations. Examination of bees against *Nosema apis* infestation and *Acarapis woodi* invasion was done according to the methods recommended by OIE (22).

Antimicrobial sensitivity testing. The test was done by a disc method on Wellcotest sensitivity test agar. From 18 hr culture on malt extract-yeast extract agar with 1% of glucose, each isolate was diluted in 0.9% sodium chloride solution to a final concentration 10^{-3}. A volume of 100 µl of each suspension was used to inoculate Mueller Hinton agar plates. The isolates were classified as susceptible, intermediate or resistant by using the zone diameter standards (Wellcotes, Wellcome) after 24 hr incubation at 35°C. A standard strain, *Escherichia coli* ATCC 25922, was used as a quality control.

Infectivity test. Test for pathogenicity of *E. faecalis* isolates was done using worker bees from healthy bee colonies. Caged bees were surface sprayed with *E. faecalis* suspension (about 2×10^4 viable cells from an overnight broth culture/bee) or fed sugar syrup with bacteria (10^7 bacterial cells/ml) for 24 hr after 8 hr of starvation. Bees were reared at an incubator at 34 °C and inspected for clinical symptoms and number of dead individuals. Treatments were done in groups, each of 20 workers. As a control served bees sprayed with or fed on sugar syrup only and reared at an incubator at 34°C. Multiplication of *M. faecalis* in body of bees artificially infected was monitored just after insect death. Insects were thoroughly rinsed in sterile distilled water, blended in Ringer solution (10 ml), homogenates were diluted serially and inoculated on malt extract-yeast extract agar containing 1% glucose to detect *M. faecalis*.

In a similar manner worker bees were treated with *Providentia rettgeri* isolates.

RESULTS

Gram-positive, catalase-negative cocci, mainly ovoid, lacking obvious capsules predominated in the cultures of the content of intestines of dead bees and in a sample of feces from sick colonies. This bacterium had grown in a pure culture from bee muscles. On the basis of biological and biochemical properties (Table 1) all isolates of cocci were classified to *Enterococcus faecalis* (20).

Tab. 1. Biological and biochemical characteristics of *E. faecalis* isolated from sick bees, *Apis mellifera* L.

Characteristics	
Serological group	D
Catalase production	negative
Type of hemolysis	α
Growth at 10°C	+
Growth at 45°C	+
Growth in: 6.5% NaCl	+
0.04% tellurite	+
0.01% terazolium	+
0.1% methylene blue milk	+
broth at pH 9.6	+
Arginine hydrolysis	+
Acid from: arabinose	–
melibiose	–
lactose	+
sucrose	+
glycerol	+
sorbitol	+
mannitol	+
esculine	+
starch	–
galactose	–
maltose	–

E. faecalis bacteraemia developed in bees fed or sprayed with the pathogen. *E. faecalis* was reisolated in pure culture from hemolymph, muscles and the alimentary tract of experimentally infected bees. The bees died during 7 days since infection with the symptoms of heavy diarrhea. A cumulative mortality of bees ranged from 18% to 72%, and it was higher after oral infection than after spraying of insects (Fig. 1). From 92 to 96% of control bees treated with sugar syrup alone, by oral route or by spraying, survived and neither diarrhea of *E. faecalis* bacteraemia were found in dead specimens.

The bacterium *E. fecalis* was found in all specimen bees and frequently it was the only organism present. Accidental microflora was not numerous. It was represented mainly by *Providentia rettgeri* and *Candida albicans*. Neither *P.*

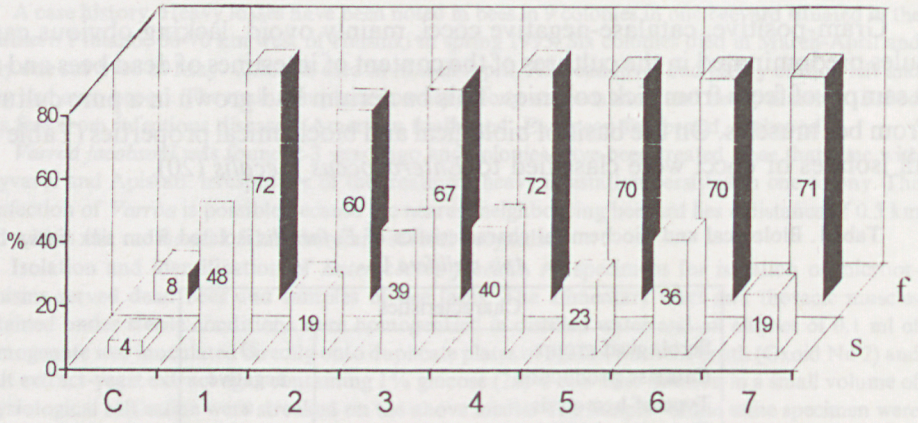

Fig. 1. Cumulative mortality (%) at day 7 of bees experimentally infected (f – feeding, s – spray) with *E. faecalis*

rettgeri nor *C. albicans* was found in muscles of the bees from sick colonies or in the hemolymph and muscles of experimentally infected bees. Mortality after oral infection or bacterial spray did not exceed 4-7%.

The disc diffusion test applied to the isolates 7 showed that *E. faecalis* was highly susceptible to erythromycin, doxycycline, terramycin, clindamycin and streptomycin, less susceptible to neomycin and lincomycin. All isolates were resistant to trimethoprim, biseptol, cloxacillin, cefatoxin and 6 of 7 isolates were resistant to ampicillin and colistin.

On the examinations of sampled bees a weak *Nosema apis* infection (less than 0 spores per unit area) was found in one colony. Two colonies were heavily infested by *Nosema* 9150 and more than 300 spores per unit area). Bees were free of *Acarapis woodi* mite. Part of specimen insects carried females of *Varroa jacobsoni.*

DISCUSSION

A septicemia caused by *Enterococcus*-type of organism has not been reported yet in the honeybee (*Apis mellifera* L.). E. faecalis was found to be a common microorganism in the intestinal content, faces and muscles of bees from sick colonies with symptoms of diarrhea and weakness. The disease resulted with death. *E. faecalis* was also pathogenic for healthy worker bees. The most characteristic signs in experimentally infected insects was diarrhea, septicemia and death. All experimentally *E. faecalis* infected bees contained this microorganism, it was absent in control insects. Other microorganisms found in sick

insects such as *Providentia rettgeri* and *Candida albicans* were scant in number and were present in a small percentage of individuals (less than 10%). Therefore, it appears that the presence of *E. faecalis* in sick bees is not an accidental contamination but that this *Enterococcus* is a causative agent of the disease. *E. faecalis* does not persist in honeybee colonies by itself although is very common in the field (21), and occurs in insect populations without apparent harm to the host (7). *E. faecalis* is common secondary organism accelerating death of *S. pluton* infected bee larvae (1, 24).

Evidence for a constant symbiotic microflora in the bee is lacking. The honeybee possess an efficient system for prevention of stable bacterial contamination of the alimentary tract. Therefore, *E. faecalis* cannot establish in a great number in healthy bee colonies and start its destructive action on brood and bees. *E. faecalis* however, may infect, develop and kill bees under stress. The examined colonies were invaded both by *Nosema apis* and by *Varroa jacobsoni*; highly stressogenic agents. The parasite *N. apis* mechanically damages the midgut wall of the bee and *V. jacobsoni* mite destructs body coverings forming the point of entry for bacteria into the body cavity. Moreover, the mite impairs protective mechanisms of hemocoel of the bee, by depression of cellular and humoral immune defense mechanisms. Hence infested colonies are more susceptible to viral 2, 3) bacterial infections (14, 16, 19) and fungal infections (13). Moreover, *V. jacobsoni* is a carrier of bacterial infections to a recipient bee host (17) and, therefore, may be a vector for *E. faecalis* which is brought into the hive by bees from the field together with contaminated pollen, nectar or water. Apart from the role of stressors in pathogenesis of *E. faecalis* infection there should be brought in mind the fact that only certain strains of this bacterium are capable of producing disease. Pathogenicity may prove to be peculiar only to certain serotypes (6). There may exist a possible analogy between *Escherichia coli* in man and *S. faecalis* in insects. Both are normal inhabitants of intestine of man, and in the case of *E. coli* a very small number of serotypes are enteropathogenic.

Tetracyclines are commonly used in practice in many countries to control American foulbrood. Because of a high susceptibility *E. faecalis* isolates to terramycin the use of this form of tetracycline in affected bee colonies is advised. Erythromycin, doxycycline, clindamycin and streptomycin were also highly effective in inhibiting the growth *in vitro* of all isolates tested. Their use in bee therapy, except streptomycin, is prohibited in most countries.

Diagnosis of *Enterococcus septicemia* should be developed upon isolation of the causative agent which is Gram-positive catalase-negative *Enterococcus faecalis* (serological group D). Clinical signs such as diarrhea, weakness and heavy losses of bees are not characteristic of *E. faecalis* septicemia. They are found in many microbial diseases, food and chemical intoxications of bees.

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STRESZCZENIE

Badaniom mikrobiologicznym poddano treść jelit, mięśnie oraz kał pszczół pochodzących z pasieki usytuowanej w Finlandii (około 70 km na zachód od Helsinek), w której wiosną 1995 roku masowo padały pszczoły. Rodziny zawierały czerw i były obficie zaopatrzone w pokarm. Główne objawy to osłabienie rodzin i silna biegunka. Z 9 rodzin tylko 1 przeżyła do maja.

Enterococcus faecalis (*Streptococcus faecalis*) dominował w przewodzie pokarmowym i kale badanych pszczół. Ten drobnoustrój wyosobniono w czystej hodowli z mięśni tułowiowych. Ponadto z około 10% pszczół izolowano niewielkie ilości *Providentia rettgeri* oraz *Candida albicans*. Przypuszcza się, że czynnikiem predysponującym do wystąpienia posocznicy była inwazja *Varroa jacobsoni* i *Nosema apis*. *E. faecalis* powodował zachorowanie i padanie pszczół robotnic zakażonych doświadczalnie *per os* lub w formie oprysku.

Szczepy *E. faecalis* były w pełni wrażliwe *in vitro* na erytromycynę, doksycyklinę, terramycynę, klindamycynę i streptomycynę, mniej wrażliwe na neomycynę i linkomycynę, oporne na trimetoprim, biseptol, cloksacylinę i cefatoksin. Sześć z 7 izolatów *E. faecalis* było opornych na kolistynę i ampicylinę.

W leczeniu posocznicy na tle *E. faecalis* zaleca się stosowanie terramycyny. Biegunkę i osłabienie rodzin nie można uznać za objawy patognominiczne dla posocznicy na tle *E. faecalis*. Zmiany chorobowe mogą występować we wszystkich tkankach, dotyczą najczęściej mięśni. O ostatecznym rozpoznaniu posocznicy wywołanej przez *E. faecalis* decyduje izolacja i identyfikacja tego drobnoustroju.