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KATEDRA EPIZOOTIOLOGII I KLINIKA CHORÓB ZAKAŹNYCH WYDZIAŁU WETERYNARYJNEGO AR W LUBLINIE
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Sacbrood of the Honey Bee of an Atypical Course. Identification of the Virus

Choroba woreczkowa o atypowym przebiegu. Identyfikacja wirusa

The honey bee colony comprises thousands of individual insects including brood and imagoes, living together in close proximity. Such conditions provide an ideal environment for the establishment and spread of infectious agents. Honey bees, like virtually all living organisms, are susceptible to viruses that may have harmful effects on normal colony and its productivity. Several viruses are known to be associated with honey bees all over the world (5, 9). Sacbrood virus seems to be the only one, out of those so far identified, to attack the larval stage of the bee, not the adult. In 1964 B a i l e y at al. (2) first proved the virus was involved in sacbrood.

Diagnosis of sacbrood of a typical course is not difficult to establish because the signs of the disease are characteristic. Diseased larvae fail to pupate after they have been sealed. Ecdysial fluid accumulates between the body of the sick individual and its unshed cuticle and the larva changes from its usually pearly white to a pale yellow and finally to a dark brown. It may have spottled or mottled appearance. The body wall of larvae dead of sacbrood becomes toughtened. It appears bloated and may be easily removed intact from the cell. When removed from the cell the sick larva is sac-like in appearance. The body content is watery and containing many fine granules. Finally, the larva dry to a flattened gondola-shape scale which can easily be removed from the cell.

In 1990 and 1991 a disease of brood clinically resembling sacbrood has appeared in apiaries in Finland and Poland. Clinical symptoms and pathological lesions of the disease were not characteristic of any bacterial or mycottic disease of the brood. Opposite to a typical sacbrood, sick larvae have not a sac-like appearance when removed from the cells and their content does not liquefy. However, dead brood heads are darker than the rest of the body, somewhat shrivelled and raised, transformed to flattened dark scales easily removed from cells of the comb. The disease appeared in colonies of a high productivity free of other infectious brood diseases. Only in some colonies in Poland *Varroa jacobsoni* of a low intensity of invasion was noted. New outbreaks of the disease were diagnosed since early spring to late summer. The disease attacked from 10 to about 20% of colonies in large apiaries and almost all colonies in small ones (up to 10 hives). The pathological conditions were not harmful for life of bee colonies. Dead brood was removed from their cells and self cure was mostly obsérved. The percentage of sick brood in a colony varied from 10 to 22% of worker larvae.

MATERIALS AND METHODS

S i c k b r o o d. Honey bee larvae revealing all stages of disease (Fig. 1) picked up from colonies in Finland and Poland served as diagnostic material. The larvae usually died after cells had been caped, mostly at the stage of upright larvae; dead larvae were extended along the floor of the cell with heads slightly raised. Their coloration turned from white to yellow, light brown, dark brown or even completely black. Cells with sick brood were scattered among capped and uncapped cells containing healthy brood. Sometimes numbers of neighbour cells

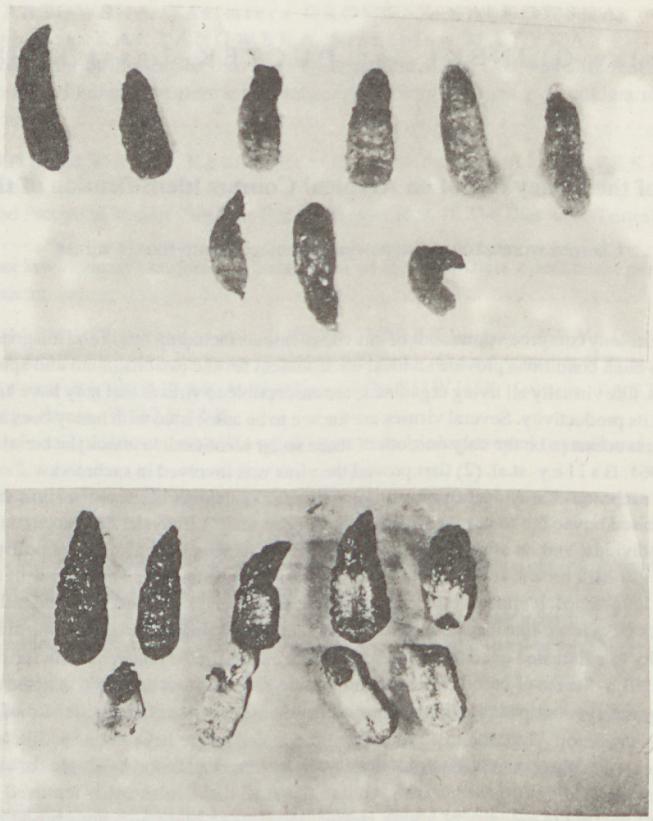

Fig. 1. Lesions appearing in successive stages of sacbrood of atypical course
Zmiany występujące w kolejnych fazach choroby woreczkowej o atypowym przebiegu

contained sick and dead individuals. The cuticle of larvae became though and leathery as the disease progressed. The dry larvage remains have easily been removed from cells. Only occasionally (mainly in Poland) larval scales have a gondola-shape.

Electron microscopy. For examinations under an electron microscope thin sections of tissues of larvae showing symptoms of a disease and larvae without any signs of sickness derived from healthy colonies (controls) were prepared. The sections were then immersed in 1–2 drops of distilled water and after a few minutes the preparates from the surface of water drops were made for electron microscope examinations. The preparates were negatively stained with phosphotungstic acid and examined for the presence of viral particles, shape and diameter of virions under the magnification of 44000 and 60000 x.

Virus propagation in honey bee larvae. The disease was transmitted to 2–3 days old honey bee larvae using crude homogenates prepared from dead brood containing a great number of viral particles visualized under an electron microscope. The homogenates were obtained by grinding 5–6 scales with 2 ml of water in a glass tissue grinder. After removal of visible particles by centrifugations, supernatant was mixed with an equal volume of honey with polled added and used to infect larvae perorally. Each larva was given 5 µl of the prepared extract which was placed with the Hamilton's syringe into the bottom of the cell. After infection the larvae were incubated (about 120 individuals in a piece of comb) with 35–40 young worker bees in small woodden-wire net cages in darkness. At the age of 4.5–5.0 days of life larvae were artificially capped with a wax foundation. Samples of larvae for an electron microscopic examinations were collected at 24 h intervals post-capping to examine their shape, colour, position in the cell and to show viral particles in larval tissues under an electron microscope.

Resistance of the virus to chloroform and heat. A fundamental feature of viruses belonging to the *Picornaviridae* family is their resistance to chloroform or aether. An aliquot of 1.0 ml of a crude extract of the larvae containing viral particles was treated with 10% chloroform for 18 h. Another sample of a crude extract prepared from the same material (1.0 ml) was heated at boiling water bath for 5 minutes to destroy infectivity of the virus. The treated extracts after mixing with an equal volume of honey with pollen added were used to infect 2–3 days old bee larvae.

Serological examination. A conventional gel double diffusion test was done to identify serologically viral particles in extracts of sick and artificially infected bee larvae. 0.75% agarose in 50 mM potassium phosphate buffer (pH 6.7) containing 5 mM EDTA was used (1). A specific antiserum for sabrood virus (end point 256) was obtained from the Rothamsted Experimental Station (B.V. Ball), England. One set of agarose wells was filled with the larval extracts, another set with anti-sacbrood antiserum. The extracts were prepared by grinding 2–3 larvae in an equal volume of potasium phosphate buffor in a tissue grinder. The homogenates were then centrifuged at 9000 or 30 minutes and a clear supernatant was poured into the wells. The presence of precipitation arcs was read after 18 h incubation in a moist chamber at room temperature.

Bacteriological examination. Samples of scales after grinding with a sterile salt saline were cultivated on Bailey's agar and blood agar to detect bacteria infecting brood. The inoculated media were incubated at 32°C for 3–4 days.

RESULTS

Tissues of naturally infected honey bee larvae contained viral particles of a cubic shape and diameter of about 30 nm. They were well visualized under an electron microscope after staining with phosphotungstic acid. The viral particles were either randomly distributed or they formed arrays in tissues. Apart from typical viral particles empty capsides were also found (Figs. 2 and 3). Virions of identical shape and diameter were found in tissues of dead larvae experimentally infected with extracts of dead brood and with extracts of brood treated with chloroform. Neither heated nor control samples induced in tissues of experimentally infected larvae formation of virions.

The disease was easily transmitted to healthy coiled bee larvae by extracts prepared from naturally infected and naturally infected and chloroform treated brood. Brood died after sealing. The colour of larvae changed from pearly white to yellowish brown and black as infection progressed; part of larvae was removed by worker bees from their cells. Unremoved brood transformed into black scales which do not adhere to the cells. A few larvae formed typical for sacbrood sac-like appearance and then transformed to gondola-shape scales. Larvae experimentally infected with heated or chloroform treated samples or samples prepared from control larvae survived experimental infection and transformed into normal bees.

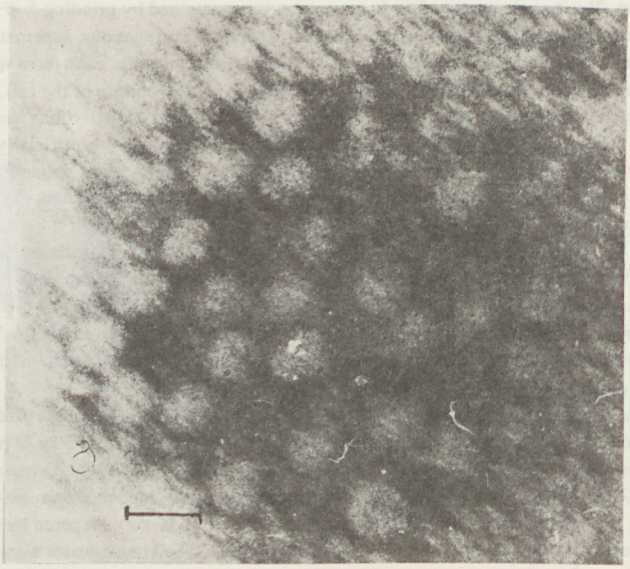

Fig. 2. Electron micrograph of virus in tissues of honey bee larvae stained negatively with phosphotungstic acid. Bar l = 38.5 nm

Mikrofotografia elektronowa wirusa w tkankach larw pszczół miodnych barwionych negatywnie kwasem fosfowolframowym

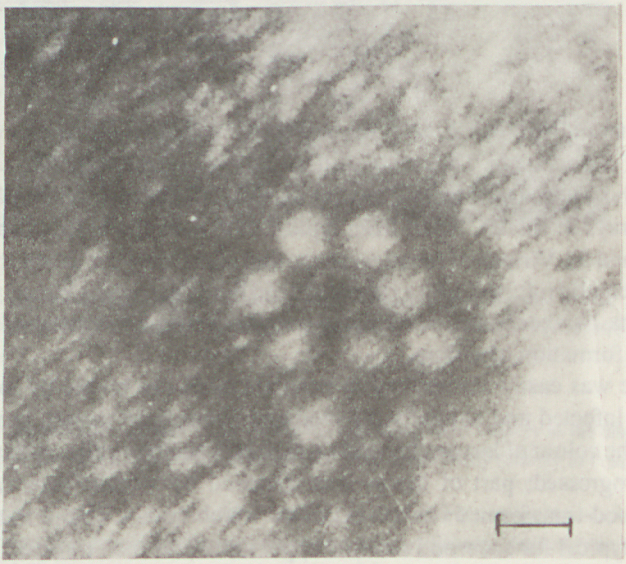

Fig. 3. Micrograph of phosphootungstic acid stained preparates of virus in larval tissues. Note empty virions (a). Bar l = 38.5 nm

Mikrofotografia preparatu wirusa w tkankach larwowych barwionych kwasem fosfowolframowym. Puste wiriony (a)

Diagnosis of sacbrood based on the results of an electron microscope examinations and bioassay experiments was confirmed by a precipitation test in agarose gel. Both extracts prepared from naturally and experimentally infected brood formed in the test after 18–19 h one, sharp and delicate precipitation arc with the sacbrood antiserum at a concentration of 1:20 and 1:40.

Results of bacteriological examinations were negative. Only in one case from scales harvested in one colony in Poland, aerobic streptococci were isolated in a small number.

DISCUSSION

Much evidence suggests that a wide variety of specific viruses are perpetuated as inapparent infections and that viral diseases are complicated by secondary bacterial infections. In sacbrood *Bacillus larvae, Melisococcus* pluton and many other bacteria, are secondary invaders in diseased individuals and hence the atypical course of sacbrood is noted. Parasitic invasions that predispose of brood to viral infections may also affect the course of sacbrood. The role of *Varroa jacobsoni*, an ectoparasitic stenophagous mite, as a vector and inducer of virial infections in invaded bee colonies is known (3). Viruses enter the haemolymph throughout wounds made by parasitizing mites. The parasite is also capable of transmitting viruses to healthy individual (4, 10). Destruction of larval tissues and impairments of immune defence mechanism of the insect host by parasitizing mites can also induce the development of latent viral infections (10). In the described outbreaks of sacbrood, *Varroa* infectation at a low intensity of invasion, was found only in Poland. Moreover, all the examined samples, except one, were bacteriologically negative. Therefore, the atypical course of sacbrood observed in outbreaks in Finland and Poland may reflect either differences in pathogenic properties of the causative virus or the presence of some yet unknown factors that prevented liquefaction of the body content of diseased brood and formation of a sac-like appearance of sick larvae.

A very useful for a diagnosis of viral diseases is detection under an electron microscope of viral particles in tissues of sick and dead insects. The description of their basic characteristics as a cubic shape and diameter of virions complemented with such parameter as resistance of virions to chloroform enabled to classify the viruses causing the described outbreaks of disease in bee brood to the *Picornaviridae* family (7).

The fact that out of many viruses attacking bees only sacbrood virus is pathogenic for bee larvae but not for adults (2, 6) and that it belongs to *Picornaviridae* made possible a tentative diagnosis of sacbrood on the basis of an electron microscope examinations, resistance of viral particles to chloroform and pathogenicity of the virus to coiled bee larvae afteroral infection. Full diagnosis, however, included a detection of a specific antigen for sacbrood virus by immunodiffusion test.

CONCLUDING REMARKS

1. Outbreaks of a disease of sealed brood of the honey bee, *Apis mellifera* L., noted in Finland and Poland are caused by sacbrood virus. An electron microscopy negatively stained with phosphotungstic acid specimens of dead brood showed characteristic viral particles.

2. Shape and diameter of viral particles, their resistance to chloroform and pathogenicity of the virus to bee larvae make possible a tentative diagnosis of sacbrood basing on an electron microscopy, physical and biological properties of the virus.

3. Further studies are needed to explain the cause (s) of atypical course of sacbrood and to determine quantitatively virulence of virial isolates.

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STRESZCZENIE

W 1990 i 1991 r. w pasiekach usytuowanych na terenie Polski i Finlandii wystąpiły masowe zachorowania czerwia krytego przebiegające wśród objawów zbliżonych do choroby woreczkowej. Różnice dotyczyły braku upłynnienia tkanek martwego czerwia. Ponadto tylko w pojedynczych przypadkach martwy czerw przypominał swoim wyglądem "gondolę".

Występowanie cząsteczek wirusa o średnicy około 30 nm i kształcie sześcianów w preparatach sporządzonych z martwego czerwia metodą "kropli wody" i wybarwionych kwasem fosfowolframowym oraz odporność wirusa na działanie chloroformu wskazują na jego przynależność do rodziny *Picornaviridae*. Do tej rodziny należy wirus choroby woreczkowej, jedyny wirus patogenny dla czerwia pszczoły miodnej. Wirus wywoływał zachowania u larw w wieku 2–3 dni po peroralnym zakażeniu wyciągiem sporządzonym z tkanek zamarłych larw. Odczyn immunoprecypitacji w żelu agarozy z użyciem swoistej surowicy odpornościowej dla wirusa choroby woreczkowej potwierdził występowanie choroby woreczkowej czerwia.

Wstępne rozpoznanie choroby woreczkowej czerwia jest możliwe na podstawie badania martwego czerwia w mikroskopie elektronowym metodą "kropli wody" uzupełnionych określeniem właściwości fizykochemicznych wirionów oraz chorobotwórczością wirusa dla czerwia pszczoły miodnej. Decydujące znaczenie w rozpoznaniu odgrywają wyniki odczynu immunoprecypitacji z użyciem swoistej dla choroby woreczkowej surowicy odpornościowej.