

THE MICROORGANISMS ISOLATED FROM THE MITES *VARROA DESTRUCTOR* AND THE VERIFICATION OF THEIR PATHOGENITY

J. HRABÁK

Luční 255, 338 28 Radnice, CZECH REPUBLIC, E-mail: hrabakj@seznam.cz

Abstract

Our research was concentrated on the finding one of *Varroa destructor* mite with pathological symptoms and on the isolation of its microorganisms. The dead mites were collected from sticky boards in beehives and observed under the stereomicroscope. The mites suspected of dying caused by pathological process were examined by means of bacteriological and mycological methods. The pathogenity of isolated microorganisms was verified in the following tests. Females with black formations in the gut's area and with white colored mycosis in the idiosoma and on its surface were found during the research. The bacteria *Enterobacter cloacae*, *Staphylococcus albus haemolyticus* and the fungi *Aspergillus flavus*, *Penicillium multicolor* and *P. simplicissimum* were isolated from the individuals with black patches and the bacteria *Enterobacter cloacae* and the fungi *Mucor ramosissimus*, *M. indicus* and *M. hiemalis* from the mites with mycosis. The laboratory tests for verification of pathogenity of isolated microorganisms were performed in laboratory cages with 40 bees and 15 females of mite *Varroa destructor*. The laboratory cages with bees infested by *Varroa destructor* were sprayed by an inoculum and by sterile saline (in control cages). The experiments proceeded at the temperature of 35°C. The laboratory tests proved the pathogenity only in bacteria *Enterobacter cloacae*, which constrained the mortality of mites average of 77,4 p.c. in the laboratory cages. The mortality in the control cages was average in 15,9 p.c. . The statistical difference with the control cages by the tests of other microorganisms was not proved. The mites infested by *Enterobacter cloacae* died with characteristic pathological changes in Malpighian tubules (macroscopically observed as the enlargement of these organs and of the idiosoma) and the membrane between genital and sternal or metapodal shields usually burst. However, the black patches were not observed in our laboratory tests.

Introduction

The search for preparations of biological control of *Varroa destructor* can be concentrated on:

- 1) Testing of microorganisms with demonstrable pathogenity for another genus of mites.
- 2) Using predaceous mites attacking store room mites.
- 3) Finding of mites with infectious symptoms in hives.

We concentrated our research on looking for mites with pathological and infectious symptoms from debris and from bee- larvae. However, we are restricted on bacterial and fungal pathogens.

Pathogenic microorganisms, described in other acarines (Laelapidae, Iphiopsidae) and ticks (Ixodida, Holothyrida), can be tested as biological control agents of varroasis, because these organisms are related to acari Varroidae.

The research had been concentrated on host of mite *Varroa jacobsoni* - *Apis cerana* accustomed, but the research of ANDERSON and TRUEMAN (ANDERSON et al., 2000) demonstrated that the *Varroa jacobsoni* is a diverse species from *Varroa destructor*, infesting honey bee colonies - *Apis mellifera*. This fact point to necessity search for pathogens by particular species of varroa.

Prospective pathogens to mites *Varroa destructor* can be divided into groups, according to taxonomy of microorganisms: nematodes, protozoa, viruses, rickettsiae and fungi. We refer to most meaningful potential pathogens in the resulting texts.

Viruses could be advantageous agents, because the viruses invade, usually, standard groups of identical cells by infection. This factor affected pathogenity for restricted organism that the pathogenity of *Varroa* pathogens for honey bees is not presupposing. The first disadvantage of viruses is difficult cultivation of big quantity of culture. The viruses are cultivated mostly *in vivo*.

Polydnaviridae, Ascoviridae and Baculoviridae are specific pathogens for arthropods (CHANDLER et al., 2001). Baculoviruses are a characteristic group for biocontrol (MARTIGNONI, 1984). They infested the gut and penetrate trough epithelial cells in the organism.

Virus-like particles were found in the fat body by mites, that infested *Apis mellifera* colonies, but the transmission tests of this particles failed. The mites with diagnosis of virus-like particles have black colored changes in the gut's tissue and fat body. (KLEESPIES, 2000)

A putative iridovirus was isolated from varroa mites from the honey bee colonies in the USA, although its pathogenity to the mites was not determined (CAMAZINE et al., 1998).

Rickettsiae were found in mites and ticks in high concentrations; however, they can be dangerous to humans and other vertebrates, too. The next disadvantage is difficult mass production, therefore the rickettsiae are not to classify as potential pathological agents, as viruses.

An unidentified rickettsia-like organisms was found in the rectum of varroa (LIU et al., 1988). These organisms were discovered in all stages of development.

Bacteria are stepping into pathogens of insects. The characteristic families of entomopathogens are Bacillaceae, Enterobacteriaceae and Streptococcaceae. Pathogenic effect of Bacillaceae is usually created by synthesis of toxin that is forming by sporulation of microbes.

Bacillus thuringiensis is used in biological control in high gauge. For example, it is applied by control of *Galleria melonella* L. in beekeeping. The *thuringiensis* killed adults and larvae of the *tetranychid* mites (HALL et al., 1971) and some species of *Mesostigmata* and *Prostigmata* (CHANDLER et al., 2001).

The strains of *Bacillus thuringiensis* were isolated from the gut of *Varroa d.*, but its pathogenity is not known (GLINSKI et al., 1990).

However, bacteria are not characteristic pathogens of mites, but their cultivation is usually situated between 30-35°C, that is characteristic for bee brood. The humidity in bee colonies is suitable for growth of bacteria, too.

Fungi are described as one of first pathogens of arthropods in history. Optimum temperature is situated about 25°C (BIRCHER et al., 1990). They could be useful to application in honey bee colonies just in winter. Only few species have optimum growth temperature about 35°C, but there exists a real attention for their pathogenity for humans, e.g. *Aspergillus*.

The mortality of fungal infection is caused by mechanical destruction on tissue, by taking off water, and by activity of mycotoxines (BIRCHER et al., 1990).

The fungi *Beauveria bassiana* has been used as mycopesticides to control of more than 700 arthropod species (GOETTEL et al., 1992). The next meaningful pathogenic fungus of insects is *Metarhizium anisopliae* (green muscardine) and other species of genus *Metarhizium*. They have a maximum growth temperature of 38 °C.

The advantage of application of fungi to biological control is very easy cultivation of mass quantity. However, the *B. bassiana*, *M. anisopliae*, can call out infection by laboratory experiment, but the infection of honey bee was not still described. (CHANDLER et al., 2001; WEISER, 1966).

The *Hirsutella thompsonii* and *Metarhizium anisopliae* were evaluated in the laboratory and in observation hives and the results were significant positive (KANGA et al., 2002)

Parasitoids The use of predatory mites depended on humidity and temperature of environment. They infested adults, development stadia, or eggs. The application of predators, which consumed the eggs, bring risk into potential intent on bee eggs, too.

Materials and Methods

Identification of pathological agents

Collection of mites

Varroa destructor mites have been collected in stand in Radnice (West Bohemia) since 1999. Beehives (*Apis mellifica* L. colonies) on the stand were equipped with bottom boards with distance gauze (mesh ϕ 10 mm) covered on the top with a separate gauze (mesh ϕ 3 mm) (Figure 1).

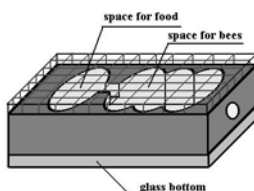


Figure 1 - Laboratory cage (schematic sketch)

Dead females got stuck on the bottom boards without being removed by bees. The stands were closely observed and dead mites were observed under the stereomicroscope. Pathological changes were expressed in the mites with conspicuous dark colour or characterised by growth of fungi on their surface.

Isolation

Dead females of *Varroa destructor* suspected of death due to pathological process were treated by ethylalcohol (70%) on the surface and then divided into two parts. One part was placed in 1 ml saline, the other part was placed in the test tube at the temperature of 4 °C for further examination. The part of mite was incubated for 2 hours in saline at the temperature of 36 °C and then the fluid was inoculated on the Petri dish and incubated at the temperature of 36 °C.

Columbia agar with 5% SB was used for bacterial culture and Sabouraud dextrose agar for fungi.

Experimental part

Experimental infections

The inoculum containing 1×10^7 cells in the mm^3 of saline with 5% glucose was sprayed on the bees infested by mites *Varroa destructor* in laboratory cages (Fig. 1). The upper part of laboratory cages was made by gauze (mesh ϕ 0,5 mm), the lower part by the glass bottom. 40 bees and 15 mites were in the laboratory cage. The bees with mites in the control laboratory cages were sprayed with the bacteria free saline with 5% glucose.

Isolation

The dead mites were taken out of the laboratory cage and were disinfected on their surface in the same way as the mites from the spontaneous fall-out. Then the puncture of idiosoms was made and the obtained fluid was cultivated and examined under the microscope.

Results

The mites exhibiting pathological black patches within their intestins were found in the debris (Fig. 2). The complex of distal and lateral intestinal diverticula were filled with black hard homogenous substance. This substance was cultivated according described methods.

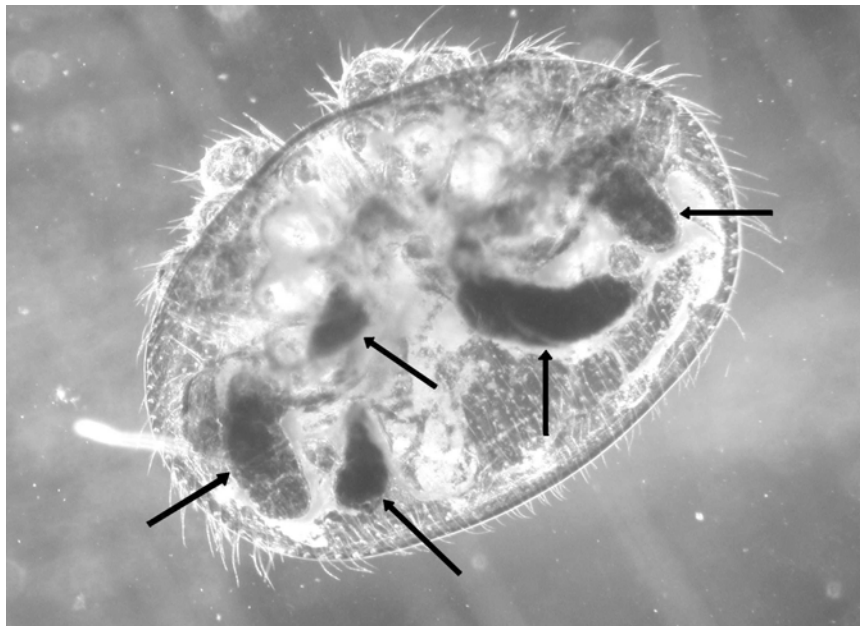


Figure II - The mite *Varroa destructor* with massive black substance in the gut's diverticuls.
(Native preparation, Stereomicroscope, Magnification: 20x)

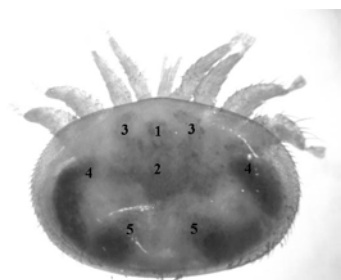


Figure 3 - The black substance in the digestive tract and black formations in the distal and lateral diverticulus of gut. 1-oesophagus, 2-proximal gut's diverticulus, 3-anterioral mezenteron, 4-lateral gut's diverticulus, 5- distal gut's diverticulus. (Native preparation, Stereo microscope, Magnification: 20x)

From these mites were isolated following microorganisms:

Fungi: *Aspergillus flavus*
Mucor ramosissimus
Mucor indicus
Mucor hiemalis
Penicillium multicolor
Penicillium simplicissimum

Bacteria: *Enterobacter cloacae*
Staphylococcus albus

The spraying of bees by inoculum prepared from isolated microorganisms proved that the *Enterobacter cloacae* is the agent, which could cause infection of the mites. The mortality was between 70-88.9%. The mites died in 48-72 hours with specific enlargement of the idiosoma. The membrane between genital ventral and sternal shield was usually broken releasing a fluid (Fig. 4). The fluid was taken from intact idiosoma with sterile hypodermic needle and the *Enterobacter cloacae* was found, microscopically as well as in bacterial culture.



Figure 4 - The pathological changes on Malpighian tubule after artificial infection, with bursting of membrane between dorsal and metapodal shields. 1- enlargement of right Malpighian tubule, 2- dorsal shield, 3- metapodal shield. (Native preparation, Stereo microscope, Magnification: 20x)

The mortality of the mites in the laboratory control cages were between 0-45% (average 15.9%). The mortality in the laboratory cages by tests of other isolated microorganisms did not prove their pathogenity:

<i>Aspergillus flavus</i>	mortality av. 22.5 %
<i>Mucor ramosissimus</i>	av. 25.2 %
<i>Mucor indicus</i>	av. 22.1 %
<i>Mucor hiemalis</i>	av. 18.6 %
<i>Penicillium multicolor</i>	av. 12.5 %
<i>Penicillium simplicissimum</i>	av. 18.6 %
<i>Staphylococcus albus</i>	av. 23.8 %

All results were statistical analyzed by means of contingency table and proved on $p > 0.95$.

Enterobacter cloacae grow in 12 hours after inoculation on Columbia agar. The colonies are round, size 2-4 mm, with thickened center and they have grey colours and mucous consistence. This bacteria is gram-negative, short rod, large 0.5-1 x 1-2 μm by the microscope following. The identification was proved during the biochemical tests (Tab. I, II). Our results were confirmed in the Czech Collection of Microorganisms (Masaryk's University, Brno), where they were used for the reidentification of this strain.

Enterobacter cloacae grow very good on the nutrient agar (1% beef extract, 1% peptone, 0.5% NaCl, 2% agar, pH 7.2), which would be used for mass production.

Table I

Biochemical activity of *Enterobacter cloacae*

	Standard results for species <i>Enterobacter cloacae</i>	Results of strain, isolated from <i>Varroa destructor</i>
Urease	d	+
Indole	-	-
Phenylalanyl-aminase	-	-
H ₂ S	-	-
VP	+	+
Gelatine (22 C)	-	-
Malonate	D	+
Dulzite	(d)	-
Raffinose	+	+
Rhamnose	+	+
Trehalose	+	+
Mannitol	+	+
Sacharose	+	+
Glukose/Gas	+	+

Symbols:
 - < 10% of strains positive
 (d) 11 to 50% of strains positive
 d 51 to 89% of strains positive
 + > 90% of strains positive

Table II

Biochemical activity of species *Enterobacter*

	Arginin-hydrolase	Lysin-dekarboxylase	Ornithin-dekarboxylase	Adonitol	Sorbitol
<i>E. aerogenes</i>	-	+	+	+	+
<i>E. agglomerans</i>	-	-	-	-	d
<i>E. sakazakii</i>	+	-	+	-	-
<i>E. gergoniae</i>	-	+	+	-	-
<i>E. cloacae</i>	+	-	+	d	+
Strain from <i>Varroa</i>	+	-	+	+	-

Symbols:
 - < 10% of strains positive
 (d) 11 to 89% of strains positive
 d 51 to 89% of strains positive
 + > 90% of strains positive

Discussion

Enterobacter cloacae was described by JORDAN in 1891. D'HERELLE described this bacteria as an entomopathogen in Yucatan peninsula in 1910. They called the bacteria as the *Coccobacillus acridiorum*. This strain caused epizootics of grasshoppers migrating in large quantities from Mexico to Yucatan and the investigators observed a black turn and the decomposition of epithelium of digestive organs of grasshoppers and their death in 8 hours after infection. The virulence of bacteria increased with successive passages. (d'HERELLE, 1911, 1912)

D'HERELLE applied bacterium isolated by him at biological control of grasshoppers in Algeria, Argentina and Tunisia in the years 1910-1912. (d'HERELLE, 1911, 1912)

During 1914-1916 SERGENT and l'HÉRITIER found it necessary to run a number of successive passages from 12 (by d'HERELLE) to 50 for them to die in 8 hours after infection. Since that time such virulent strain which d'HERELLE had at his disposal has not been isolated (WEISER, 1966).

We have demonstrated that *Enterobacter cloacae* which had been found in digestive organs of mite *Varroa destructor* is pathogen for them. Until now, this bacteria has not been cited among prospective biological control agents of *Varroa destructor* in the large review by Chandler et al. (CHANDLER, 2001).

Enterobacter cloacae is taxonomically defined as gramnegative, aerobic and nonsporulating rod. Its antigen structure is typically mosaic. Optimum growth temperature rests between 30-37°C. Temperature 100°C kills it in 2 minutes. Biochemical activity that is typical for species *Enterobacter cloacae* is mentioned in table.

REFERENCES

- Anderson D.L., Trueman J.W.H. (2000), *Varroa jacobsoni* (Acari: Varroidea) is more than one species, *Experimental and Applied Acarology* 24, 165-189
- Bircher S. et al. (1990), *Medical Microbiology*, The C.V. Mosby Company, Maning, USA
- Camazine S., Liu T.P. (1998), A putative iridovirus from the honey bee mite, *Varroa jacobsoni* Oudemans, *Journal of Invertebrate Pathology* 71, 177-178

- Chandler D., Sunderland K.D., Ball B.V., Davidson G. (2001), Prospective Biological Control Agents of *Varroa destructor* n.sp., an Important Pest of the European Honeybee, *Biocontrol Science and Technology* 11, 429-448
- Glinski Z.F., Jarosz J. (1990), Micro-organisms associated fortuitously with *Varroa jacobsoni*, *Microbios* 62, 59-68
- Goettel M.S., Johnson D.L. (1992), Environmental impact and safety of fungal biocontrol agents, in Lomer C.J. (Ed.), Biological control of locusts and grasshoppers, International Wallingford, pp. 356-361
- Hall I.M., Hunter D.K., Arakawa K.Y. (1971), The effect of the β -exotoxin fraction of *Bacillus thuringiensis* on the citrus red mite, *Journal of Invertebrate Pathology* 18, 359-362
- D'Herelle F.H. (1911), Sur une épizootie de nature bactérienne sévissant sur les sauterelles du Mexique, C.R.Acad.Sci. 152, 1413-1415
- D'Herelle F.H. (1912), Sur la propagation dans la République Argentine de l'épizootie des sauterelles, C.R.Acad.Sci. 152, 1413-1415
- Kanga L.H.B., James R.R., Boucias D.G. (2002), *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite, *Journal of Invertebrate Pathology* 81, 175-184
- Kleespies R.G., Radtke J., Bienefeld K. (2000), Virus -like particles found in the ectoparasitic bee mite *Varroa jacobsoni* Oudemans, *Journal of Invertebrate Pathology* 15, 87-90
- Lindberg C.M., Melathopoulos A.P., Winston M.L. (2000), Laboratory evaluation of miticides to control *Varroa jacobsoni* (Acari: Varroidae) a honey bee (Hymenoptera: Apidae) parasite, *Journal of economic entomology* 93(2), 189-198
- Liu T.P., Ritter W. (1988), Morphology of some microorganisms associated with the female mite *Varroa jacobsoni*, a survey by electron microscopy, in Needham E. et al. (Ed.), Africanized honeybees and bee mites, Ellis Horwood, Chichester, pp. 467-474
- Martignoni M.E. (1984), Baculoviruses: an attractive biological alternative, in Burges H.D. (Ed.), Chemical and Biological Controls in Forestry, Seattle Washington, pp. 55-67
- Weiser J. (1966) Nemoci hmyzu, *Academia*, Praha