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TOXIC ELEMENTS AS A RISK FACTOR FOR THE SURVIVAL OF THE HONEY BEES (*Apis mellifera* L.)

Milica Živkov Baloš[1*], Željko Mihaljev[1], Sandra Jakšić[1]

[1]Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia

Abstract

Toxic element pollution is an ecological concern in the regions where mining, industry and agriculture are developing. Anthropogenic impact on the environment results in the reduction of the population of honey bees worldwide with varying degrees of morbidity and mortality. Bees exposed to contaminants produce polluted products through various sources, including foraging activities on contaminated plants. Therefore, monitoring of honey bee products in terms of toxic elements is very important for food safety and for preventing potential future ecological problems. The data presented in this review are useful for bee health protection and improving the quality and safety of honey production chain.

Key words: honey bee, honey, toxic elements

[1*] Corresponding Author: milica@niv.ns.ac.rs

TOKSIČNI ELEMENTI – FAKTOR RIZIKA ZA PREŽIVLJAVANJE MEDONOSNIH PČELA (*Apis mellifera* L.)

Milica Živkov Baloš[1], Željko Mihaljev[1], Sandra Jakšić[1]

[1]Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija

Kratak sadržaj

Kontaminacija životne sredine toksičnim elementima je ekološki problem u regijama sa razvijenim rudarstvom, industrijom i poljoprivredom. Antropogeni uticaj na životnu sredinu utiče na smanjenje populacije medonosnih pčela u svetu, sa različitim stepenom morbiditeta i mortaliteta. Pčele izložene toksičnim elementima, iz različitih izvora, uključujući aktivnosti prikupljanja nektara i polena sa kontaminarinih biljaka, proizvode kontaminirane proizvode. Stoga je monitoring toksičnih elemenata u pčelinjim proizvodima važan za bezbednost pčelinjih proizvoda kao hrane, a takođe i za sprečavanje budućih ekoloških problema. Informacije u ovom radu mogu biti korisne za unapređenje i zaštitu zdravlja pčela i poboljšanje kvaliteta i bezbednosti meda.

Ključne reči: pčela, med, toksični elementi

INTRODUCTION

Honey bee (*Apis mellifera* L.) is an extremely important insect not only for humans, but also for the entire ecosystem to function. Honey bee is completely dependent on flowering plants (Roman et al., 2011). They forage over very large areas and bring plant materials (nectar, pollen, propolis and honeydew) to their hives. Toxicological conditions of the environment in which honey bees live directly affect them, since their existence is directly related to the natural environment. For these reasons, honey bees and their products are considered as good bioindicators of environmental pollution (Aljedani, 2020; Costa et al., 2019; Chicas-Mosier et al., 2017).

Environmental and food contamination with toxic elements has pronounced carcinogenic and mutagenic effects, causing poisoning and metabolism disruption (Murashova et al., 2020). Although trace elements (micronutrients) play an important role in the metabolism, they have potentially harmful effects. The micronutrient metals (zinc, Cu, iron, selenium, chromium, etc.)

can contribute to neurodegeneration, if they are outside their biologic range (Chicas-Mosier et al., 2017). In addition, combined effects of co-exposure to different metals can also occur (Monchanin et al., 2021). The non-essential elements (lead, cadmium, mercury) can be toxic even in a trace amount. Regarding their level of hazard, chemical elements are divided into three groups:

1. highly hazardous substances (arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), zinc (Zn));
2. moderately hazardous substances (copper (Cu), molybdenum (Mo), chromium (Cr), tin (Sn));
3. low hazard substances (tungsten (W), barium (Ba), strontium (Sr), manganese (Mn)) (Murashova et al., 2020).

This review is aimed at reporting the information about the impact of some toxic and potentially toxic elements on honey bee health and survival. Since honey bee products are widely used in human nutrition, and may be contaminated with toxic elements, the present review addresses the first of all apicultural and food scientists. This information should be passed on to beekeepers so that they can protect bee communities from the effects of environmental contamination and produce safe bee products.

TOXIC ELEMENTS – EXPOSURE AND TOXICITY FOR HONEY BEE

Pb is one of most widespread environmental contaminants, and its content is a subject of numerous environmental studies (Bilandžić et al., 2011). This toxic element is a natural component of the biogeosphere, and it enters the environment from metal smelters, coal-fired power plants, from sewage sludge or waste oil, or as a result of solid waste combustion. The amount of Pb from natural sources in the biosphere is small compared to anthropogenic Pb sources. However, the dominant anthropogenic emission of Pb into the environment was the result of the use of organo-Pb compounds - additives in the oil industry. Pb use in automobile fuels was forbidden a few years ago, but air and water contamination is still quite high (Lambert et al., 2012). The primary sources of Pb exposure for animals are contaminated soil, Pb paint on decrepit buildings, Pb contaminated water, and Pb based products like batteries (Waldner et al., 2002). The bioavailability of Pb in soil to plants depends on soil acidity and the content of organic matter. Higher soil acidity and lower organic matter content will cause higher Pb bioavailability. Translocation of Pb ions in plants is limited. The largest amounts of Pb are found in cell walls of root cells.

Pb is accumulated mainly inside the organs of bees feeding in industrial areas. Low levels of Pb exposure cause unspecified cardiovascular, haematological, and neurodevelopmental changes (NRC, 2005). In natural feeding areas, most of Pb is found on the surface of bee bodies (Sadowska et al., 2019). Pb negatively affects bees' immune system - it slows down appetitive learning and reduces long-term memory specificity (Monchanin et al., 2021).

Most industrially produced As originates from agricultural products such as insecticides, herbicides, fungicides, algicides, wood preservatives, and growth stimulators for plants and animals (Eisler, 1988). The use of pesticides containing As as well as other chemical products in agriculture results in As accumulation in soil and plants and consequent finding of As as a trace element in the environment and food (Roy and Saha, 2002; Mandal, 2017). As causes poisoning that is associated with stomach pain. It is a protoplasmic poison. When consumed by insects, arsenicals are absorbed through the midgut or ventriculus wall. The toxicity of As is attributed to tissue and epithelium disintegration and protein precipitation. Poisoned honey bees display the symptoms such as inability to fly, distended abdomens, and diarrhoea. Honey bees often die of poisoning during foraging. The As source for nonforaging adult bees is contaminated pollen, nectar, and water brought by foragers. The majority of bees that die in the hive are nurse bees (Fujii, 1980).

The concentration of Cd in the environment increases significantly due to natural sources (volcanic activity, weathering and erosion) and anthropogenic activities (mining, smelting, industrial production of plastics, dry batteries, paints, and also through phosphate fertilizers that contain significant amounts of Cd) (Polykretis et al., 2016; Satarug et al., 2003). Cd is actively absorbed by plant roots, it is transferred into nectar and pollen, and it subsequently accumulates in pollinators and their products (Bogdanov, 2006). Polykretis et al. (2016) showed in their study that Cd causes a reduction in immunocompetence in 3 days following the exposure to Cd in honey bees.

The impact of the elements like Hg, aluminium (Al), Cr, nickel (Ni) and other potentially toxic elements on bees has been studied much less. Al is present in the soil in variable concentrations. Bioavailability of Al to organisms is increasing through mining activity, soil acidification, and carbon emission. In acidic environments, it can be a major limiting factor to many plants and aquatic organisms. Al exposure may be detrimental to foraging bee behaviours and to other ecologically relevant behaviours. Chicas-Moiser et al. (2017) showed that Al affects floral decision making of bees potentially by altering sucrose perception, increasing the activity level and reducing the likelihood of foraging on uncontaminated resources. Selenium (Se) is a micronutrient

and an essential mineral to plants and animals. This element is naturally found in alkaline lands, but agricultural water dissolves it, leading to a build-up of selenates, the bioavailable form of Se. Hladun et al. (2012) concluded that the bees fed selenate were less responsive to sucrose. It is possible that this will lead to a reduction in incoming food resources in the hive. Besides, if honey bees forage on nectar containing Se, reduction in population numbers may occur, due to direct toxicity. The same authors stated that it is possible that honey bees cannot detect detrimental concentrations of Se.

During foraging activities bees are exposed to pollutants. Their hairy bodies can keep different particles from the atmosphere, soil and water, and therefore, the toxic element levels in honey may reflect the actual amount in the environment (Costa et al., 2019; Islam et al., 2014; Lambert et al., 2012; Porrini et al., 2003; Przybyłowski and Wilczyńska, 2001). In some cases, ultra-fine particles of metal are inhaled by honey bees when they fly (Borg and Attard, 2020). However, bees can be exposed to contaminants through ingestion of contaminated pollen and nectar (Di et al., 2016).

Honey bees may be exposed to metal pollution found in an area of around 7 km^2 surrounding the hives (Đogo Mračević et al., 2020). Contamination of honey bees and their products by toxic elements may be a result of the industrial development, urbanization and transport (Hamad et al., 2020; Tutun et al., 2019; Lambert et al., 2012; Bilandžić et al., 2011). In addition to the above-mentioned sources, contamination of honey may be caused by the use of wrong procedures during harvesting, fumigation, extraction and processing, storage and conservation phases (Bartha et al., 2020).

Toxic elements from the soil accumulate in plants and migrate to the body of bees and reach the consumers through honey products and food chain. It is known that plants accumulate toxic elements not only from the soil but from the air as well. The type of plant, the mobility and bioavailability of toxic elements affect transfer from soil to flowers or other parts of the plants visited by foraging bees. Herbaceous plants of natural biocenoses have a greater potential to accumulate toxic elements compared to agrocenoses (Murashova et al., 2020). It is important to point out that the literature clearly shows that sunflower can accumulate toxic elements (As, Pb, Cu, Cd, Ni, Cr, Co), mainly in shoots and roots (Dhiman et al., 2017; Stoikou et al., 2017; Angelova et al., 2016; Garcia et al., 2006). Since growing sunflower plants has a high potential to collect metal contaminants, they are considered "hyperaccumulators" of metals (Dhiman et al., 2017).

In addition to affecting plant health, survival and productivity, environmental contamination exposes pollinators that depend on these plants to po-

tentially toxic levels of toxic elements. The sublethal accumulation of some metals and metalloids in the pollen and nectar of flowering plants can have a significant effect on pollinator health and survival (Burden et al., 2019). Some studies confirm that bees have the ability to detect some contaminants through receptors on antennae and proboscis (Burden et al., 2019; de Brito Sanchez, 2011). Foraging bees do not detect some toxic elements, like Cd and Se, they may readily consume them and therefore have a major effect on the health and survival of the colony (Hladun et al., 2012; Burden et al., 2019). Still, honey bees can reject contaminated food, the toxic levels of metals and metalloids in the environment are still a significant risk to pollinators.

During foraging activities, worker bees partially purify raw material for honey production from contaminants. That is why there is a lower level of toxic elements in products than in raw material for honey production (Roman et al., 2011). According to the research conducted in different countries, the concentrations of toxic elements in honey and honey bee products decreases in following order: bee > pollen > wax > propolis and nectar > honey (Roman et al., 2011; Lambert et al., 2012; Formicki et al., 2013; Aljedani, 2020; Gutiérrez et al., 2020). The metals accumulate over time in the bees' organs and the nest, leading to toxic effects on the larvae and older bees (Aljedani, 2020; Burden et al., 2019; Sadowska et al., 2019). Contaminants also accumulate in the hive. Di et al., 2016, Formicki et al. (2013) showed in their study that Cd accumulates the most in beeswax, while Pb content was high in both wax and honey. Exposure of honey bees to As, Cu or Pb reduces learning and memory performances (Monchanin et al., 2021). Larvae are much more sensitive to the sublethal and lethal concentrations of metals than adult bees, and they exhibit significantly increased mortality (Burden et al., 2019).

TOXIC ELEMENTS IN HONEY BEE PRODUCTS

Honey bee products are regarded as natural, healthy and clean (Bogdanov, 2006), but the public is generally not informed about the fact that honey may also contain substances that could be toxic. Honey is a natural food with nutritional, sensory and potentially therapeutic properties consumed without any processing and is characterized by complex composition (Sergalio et al., 2019). Honey has been recognized as a source of energy in human nutrition (Boussaid et al., 2018) because its sugars are easily digestible (El Sohaimy et al., 2015) and it is widely used as a sweetener in food industry and in a large number of food products (Amiry et al., 2017; Kek et al., 2017). These properties are related to the chemical composition of honey. The possible health benefits of consuming honey and bee products have been documented in early Greek,

Roman, Vedic, and Islamic texts and the healing qualities of honey were addressed by philosophers and scientists all the way back to ancient times (Prica et al., 2015).

The major component of honey is sugar, although other minor components, such as enzymes, proteins, organic acids, vitamins, minerals, pollen grains, waxes and phytochemicals, are also present (Buba et al., 2013; Sousa et al., 2016; Kek et al., 2017; Živkov Baloš et al., 2019).

The properties and composition of honey depend on the region, bee species, production season, floral source, soil characteristics, the period when it is stored in the honeycomb, method of harvesting and postharvest storage (Živkov Baloš et al., 2020). Mineral concentrations in honey depend on botanical origin, climate conditions, and significantly on geographical origin and type of soil in which the plants grow (Živkov Baloš et al., 2018; Lazarević et al., 2017; Uršulin-Trstenjak et al., 2015; Formicki et al., 2013). The minerals mainly originate from the soil and nectar-producing plants, but they may also come from anthropogenic sources, such as environmental pollution (Solayman et al., 2016). Solayman et al. (2016) listed 54 minerals reported to be found in honey in the literature on honey published in the past 15 years, from all over the world. The mineral content of honey contributes to the colour of the honey. Darker honey types are richer in minerals. Black locust and sunflower honey are characterised by low concentrations of ash and minerals, compared to meadow, chestnut and honeydew honey (Lasić et al., 2018; Dhahir and Hemed, 2015; Uršulin-Trstenjak et al., 2015). Electrical conductivity (EC) is closely related to the concentration of minerals. Lighter honey types are characterized by a lower EC than the darker ones (Živkov Baloš et al., 2018; Živkov Baloš et al., 2019a).

Honey and other bee products can be a useful indicator for assessing environmental pollution (Živkov Baloš et al., 2021; Đogo Mračević et al., 2020; Sergalio et al., 2019; Lazarević et al., 2017; Moniruzzaman et al., 2014; Bilandžić et al., 2011). Traffic–related pollution and chemical intensive agriculture contaminate the air, water, and soil, which also leads to the increasing levels of some toxic elements in honey bee products. The increased levels of some toxic elements were found in close proximity to industrial areas (Bartha et al., 2020; Hamad et al., 2020; Sadowska et al., 2019; Formicki et al., 2013; Bilandžić et al, 2011). Pb is metal whose maximum content in honey is limited by regulations. The maximum permissible value of Pb is prescribed by national regulation on maximum concentrations of certain contaminants in food and it is set at 0.10 mg of Pb/kg for honey (Official Gazette, 81/2019). This regulation is harmonized with the European regulation (Commission Regulation, 1005/2015).

In our previous study, Pb concentrations ranged from 0.002 to 0.096 mg/mg with a mean value of 0.051 mg/kg in fifteen sunflower honey samples from all the examined locations in Serbia (Živkov Baloš et al., 2021). The following Pb levels have been found in the honey from different countries: Croatia (65.2 µg/kg; reported by Bilandžić et al., (2011) and 0.02 – 0.11 mg/kg; reported by Uršulin-Trstenjak et al., (2015)), Romania (51.674 µg/kg; Oroian et al., 2016), Poland (0.048 mg/kg; Przybyłowski and Wilczyńska, 2001), Turkey (0.80 mg/kg; Tutun et al., 2019), Iran (507.58 µg/kg; Aghamirlou et al., 2015), Iraq (0.108 – 0.820 mg/kg; Dhahir and Hemed, 2015), and Malaysia (0.36 mg/kg; Moniruzzaman et al., 2014). The authors of the cited articles have concluded that higher concentrations of Pb in the examined honey samples may be a result of the location of hives in the areas, as they were near roads, industrial or building sites.

CONCLUSION

The danger of honey bee extinction is a significant issue not only from the aspect of ecology, but also from the economic point of view. The accumulation of toxic metals in the pollen and nectar of flowering plants can have a detrimental effect on honey bee health and survival. Honey and honey bees are good indicators used for monitoring of environmental pollution. Mineral composition of honey bee products is strongly affected by both natural and anthropogenic factors. The data presented in this paper are useful for improving the quality of honey production chain. Beekeepers should pick the location of their hives with caution. The procedures applied during the production and processing of honey should be in accordance with hygiene standards, in order to prevent the contamination of bee products from beekeeping source. Besides, it is important to monitor the levels of toxic elements in the honey and honey bees in terms of their toxicity and for the prevention of future ecological issues.

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Author's Contribution:

M.Ž.B. drafted the manuscript and made substantial contributions to the basic idea; Ž.M. were involved in drafting of the manuscript; S.J. revised the manuscript critically.

Competing interest

The authors declare that they have no competing interests.

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DETECTION OF MICROPLASTIC RESIDUES - DEVELOPING A METHOD FOR PHTHALATES IN HONEY

Brankica Kartalovic[1*], Jelena Vranešević[1],
Jelena Petrović[1], Biljana Đurđević[1], Radomir Ratajac[1]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia

Abstract

In this pilot study, a method for the determination of phthalates in honey was developed. The following phthalates are included: dimethyl phthalate, diethyl phthalate, di-isobutyl phthalate, di-n-butyl phthalate, bis(2-ethylhexyl) phthalate, and di-(n-octyl) phthalate. For the preparation of the samples, the method of liquid-liquid extraction with hexane with an ultrasonic bath was used. The analysis of the prepared samples was performed using gas chromatography and a mass detector. The method is reliable, sensitive, and reproducible with a detection limit of 0.28 - 1.38 μg/kg. This paper presents the results of testing samples of honey stored in glass and plastic packaging for three years in order to determine the migration of phthalates. Dimethyl phthalate was not found in the tested samples stored in plastic and glass packaging. Diethyl phthalate was not found in samples stored in glass packaging while the concentration of diethyl phthalate in samples from plastic packaging was 3.34 μg/kg. The concentrations of di-isobutyl phthalate, di-n-butyl phthalate and bis(2-ethylhexyl) phthalate, determined in samples from glass packaging were 5.32, 1.32 and 4.45 μg/ kg, and in honey samples from plastic packaging 15.84, 16.01 and 14.44 μg/kg. Concentrations of di-(n-octyl) phthalate were less than the LOQ in both types of samples.

Key words: GC-MS, plastic packaging, DiBP, DBP, DEHP

[1*] Corresponding Author: brankica@niv.ns.ac.rs

RAZVOJ ANALITIČKE METODE GASNE HROMATOGRAFIJE ZA ODREĐIVANJE FTALATA U MEDU

Brankica Kartalović[1*], Jelena Vranešević[1],
Jelena Petrović[1], Biljana Đurđević[1], Radomir Ratajac[1]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija

Kratak sadržaj

U ovoj pilot studiji razvijena je metoda za određivanje ftalata u medu. Predmetni ftalati su: dimetil ftalat, dietil ftalat, di-isobutil ftalat, di-n-butil ftalat, bis(2-etilhexil) ftalat i di-(n-octil) ftalat. Za pripremu uzoraka je korišćena metoda tečno-tečne ekstrakcije sa hexanom uz pomoć ultrazvučnog kupatila. Analiza pripremljenih uzoraka je rađena primenom gasne hromatografije kuplovane sa masenim detektorom. Metoda je pouzdana, osetljiva i reproducibilna sa granicom detekcije od 0,28 – 1,38 µg/kg. U ovom radu prikazani su rezultati ispitivanja uzoraka meda koji je skladišten u staklenoj i plastičnoj ambalaži tokom tri godine s ciljem utvrđivanja migracije ftalata. Dimetil ftalat nije nađen u ispitanim uzorcima skladištenim u plastičnoj i staklenoj ambalaži. Dietil ftalat nije pronađen u uzorcima meda skladištenim u staklenoj ambalaži, dok je koncentracija dietil ftalata u uzorcima meda skladištenim u plastičnoj ambalaži bila 3.34 µg/kg. Koncentracija di-isobutil ftalata, di-n-butil ftalata i bis(2-etilhexil) ftalata, ispitanih u uzorcima meda skladištenim u staklenoj ambalaži je bila 5,32, 1,32 and 4,45 µg/ kg, a u uzorcima meda skladištenog u plastičnoj ambalaži 15,84, 16,01 i 14,44 µg/kg. Koncentracije di-(n-octil) ftalata su bile manje od LOQ u obe vrste uzoraka.

Ključne reči: GC-MS, plastična ambalaža, DiBP, DBP, DEHP

INTRODUCTION

Nowadays, plastic is increasingly used for food packaging due to the low cost of materials, its potential for thermal sealing, optical properties, and it is also suitable for making different shapes and sizes. Due to these properties, plastic products for food packaging and beverages have surpassed the use of materials such as glass or tinplate. Plastic packaging has many advantages - it is light, resistant, and easy to shape, i.e. it can be formed in different shapes and

sizes and thus adapted to different types of food, from solids to liquids. Plastic packaging provides good protection against damage. However, from the chemical point of view, when it comes to biodegradability, its harmful effects are the subject of a great number of research papers today. EU Regulation 1935 dating from 2004 is based on the fact that all materials or objects that come into direct or indirect contact with food must be inert enough to prevent the transfer of things to food in the quantities that are large enough to endanger human health or cause unacceptable changes in food composition or deterioration of its organoleptic properties. Plastic can be decomposed into compounds harmful to human health. These are divided into organic and inorganic compounds. The first group includes amines, phenols, and phthalates. In order to obtain softer and more flexible plastic products, so-called softeners are added during their production, and phthalates are the subject of this pilot study.

Diesters of 1,2-benzenedicarboxylic acid, better known as phthalates, are a group of man-made chemicals widely used in industry (Meeker et al, 2009). They are present primarily in plastic products, toys, medical instruments, industrial materials, food, and clothing. These compounds have the ability to disrupt the function of the endocrine system. The effects depend on the dose, duration of action, and developmental stage of the organism. The fetus, newborn, and children in puberty are the most vulnerable categories. Exposure to phthalates begins in the intrauterine period, as they freely pass through the placental barrier. It is believed that the side effects of phthalates can be manifested through neurocognitive disorders, allergies, asthma, testicular cancer, liver and kidney damage, insulin resistance and obesity, thyroid dysfunction, and respiratory system irritation. Phthalates in females can lead to anovulation, premature puberty, and changes in the duration of pregnancy (Butala et al, 2004; Yen et al, 2011; Bajkin et al, 2014). Bis(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) and di-isobutyl phthalate (DIBP) ('the four phthalates') are listed in Annex XIV to Regulation (EC) No 1907/2006 as substances that are toxic for reproduction, category 1B. Therefore, phthalate toxicity poses a significant risk to human health.

According to the "Rulebook on the Restrictions and Ban of Production, Placing on the Market and Use of Chemicals ("Official Gazette of the Republic of Serbia RS", 90/2013, 25/2015, 2/2016, 44/2017, 36/2018 and 9/2020) DBP, DEHP, BBP are prohibited for use in toys and objects intended for child care in concentrations higher than 0.1% of plasticized materials, while di- "isononyl" phthalate (DINP), di-„isodecyl" phthalate (DIDP), di-n-octyl phthalate (DNOP) are prohibited for use in toys and items intended for the care of children that children can put in their mouths in concentrations higher than 0.1% of plasticized materials. Given the harmful effects of phthalates on hu-

man health and the fact that they are increasingly found in plastic food packaging, there is a justified need to place clearly defined restrictions on these compounds in food packaging, and food as well. The honey whose production process does not enable contamination with phthalates, except in the case of packaging and storage in plastic packaging, is the sample whose analysis could prove the migration of phthalates in the product. Also, certain types of honey crystallize during storage- some faster and some slower. Although crystallization is a natural property of honey, a large number of consumers do not like crystallized honey, so in order to decrystallize it, they heat it, which increases the migration of phthalates. In order to prove this, it is necessary to analyse the phthalate content in honey samples. The method of liquid-liquid extraction with hexane and the analysis of the stored samples using gas chromatography-mass spectrometry (GC-MS) were chosen. The goal of this study is to develop and validate a method for the determination of phthalates in honey.

MATERIAL AND METHODS

Honey samples

For the purpose of determining the presence of phthalates in honey, ten samples of different origin were collected. Honey samples were randomly collected from two sources: honey samples that were brought to the Scientific Veterinary Institute "Novi Sad" (NIV-NS) in plastic jars and honey samples that were collected from NIV-NS's bee yard in glass jars. All the samples were in their original packaging and were transferred to the laboratory, properly labeled and stored in a dark place at room temperature for 3 years.

Reagents and materials

Standards of phthalate acid esters (PAE)" were investigated in this study, namely dimethyl phthalate (DMP; C10H10O4), diethyl phthalate (DEP; C12H14O4), diisobutyl phthalate (DiBP; C16H22O4), dibutyl phthalate (DBP; C16H22O4), bis(2-ethylhexyl) phthalate (DEHP; C24H38O4), di-n-octyl-phthalate (DnOP); C24H34O4)) they were purchased from dr. Ehrenstorfer GmbH (Germany). In Table 1, analytical data include Abbreviation, Retention time and Qualitative and Quantitative ion monitoring. N-Hexane was HPLC grade (Carlo Erba, Milan, Italy). The solutions of each phthalate were prepared at concentrations of 1 mg/mL. Phthalates solutions at different concentrations (0.005, 0.01, 0.1, 0.05, 0.5 μg/mL) were prepared by dilution in n-hexane. The solutions were stored in vials at −20 °C. In order to avoid cross-contamination

due to reagents, materials, and laboratory equipment, a thorough cleaning procedure was performed: the glassware was soaked and washed in acetone, dried at 140 °C for at least 4 h. All the solvents used in the analysis were tested in order to check the potential presence of PAE contamination using GC–MS analysis. Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, USA).

Table 1. Chemical data on the compounds investigated in this study.

Compound	Abbreviation	Retention time(min)	Quantitative ion(m/z)	Qualitative ion (m/z)
Di-methyl phthalate	DMP	7.36	163	77/194
Di-ethyl phthalate	DEP	8.44	149	177/176
Di-isobutyl phthalate	DiBP	10.78	149	104/167
Di-n-butyl phthalate	DBP	11.86	149	205/104
Bis(2-ethylhexyl) phthalate	DEHP	20.75	149	167/206
Di-(n-octyl) phthalate	DnOP	24.89	149	279/104

Sample pretreatment

The amount of 5 g of honey and 10 mL of ultra-pure water was put into a 100 mL screw-cap glass centrifuge tube with a conical bottom and vigorously vortexed to for at least 1 min in order to form a homogeneous solution. After that, the solution was mixed with 10 mL of hexane, and submitted to extraction by shaking in a mechanical shaker for 40 min. Then, the organic phase was separated by centrifugation at 3000 rpm for 10 min and collected. The sample was once again extracted with 10 mL of hexane and the above-described procedure was repeated. The two portions of supernatant were collected and transferred to a clear conical flask and evaporated to dryness at 40 °C with a rotary evaporator. The residue was dissolved in 1.0 mL of hexane and the final solution was used for GC–MS analysis (Zhou et al, 2014). The method precision was evaluated, as described by Zhou et al. (2014). The retention times of the peaks and target ions, obtained from the standard solution of phthalates served as a base point for the phthalate's determination in samples.

GC-MS Analysis and Instrumentation

The identification of phthalates was based on a comparison of retention times of the peaks and target ions with those obtained from a standard mixture of phthalates (standards supplied by instrument manufacturer). The quantification was based on external calibration curves prepared from the standard solution of each of the examined phthalates.

The GC operating conditions are shown in Table 2. The verification of the peaks was carried out based on the retention times and target ions were compared to those of external phthalates. A solvent blank was also analyzed, phthalates were detected at the concentrations lower than LOQ.

Table 2. The GC operating conditions

Descriptions	Conditions
Instrument	Agilent 7890B/5977A MSD (Santa Clara, CA, USA)
Column	Fused silica column (30 m × 0.25 μm film of HP-5M-thickness) Agilent Technologies, Inc., (Santa Clara, CA, USA)
Temperature	Injection 280 °C MSD 280 °C Column 90 °C (1 min hold) to 210 °C at 15 °C / min (hold 2 min); then at the rate of 5 °C/min to 240 °C to hold 5 min; followed by an increase of 5 °C/min to 250 °C, and the followed by an increase of 25 °C /min to 300 °C held for 4 min.
Injection volume	2 μL

The determination was performed in splitless mode. Carrier gas was Helium, velocity: 35.698 cm/sec; pressure: 7.0 psi. The determination was made at constant flow.

RESULTS

Method Validation

Method validation and quality control were conducted following the European Commission SANTE /11813/2017 Regulation (European Commission, 2017). The method was validated in terms of the optimal linearity ($r^2 > 0.99$). Precision was evaluated by repeatability in triplicate (50.0 μg/kg, n = 10) and

it ranged from 0.79 – 5.72%. Recovery ranged from 88.51% to 112.23%. The obtained results are shown in Table 3.

Table 3. The average values of LOD, LOQ, precision, linearity, recovery and RSD in honey samples, spiked with 50 μg/kg (n = 20)

Compound	LOD [1] (μg/kg)	LOQ [2] (μg/kg)	Precision (%)	Linearity (r^2 [3])	Recovery (%)	RSD [4] (%O)
DMP	1.38	4.68	4.26	0.9991	96.65	5.26
DEP	0.28	1.12	0.78	0.9992	112.23	10.20
DiBP	0.29	1.13	3.36	0.9991	94.62	12.62
DBP	0.89	2.76	3.49	0.9996	95.12	7.12
DEHP	0.59	2.07	3.48	0.9991	92.12	3.38
DnOP	1.22	3.89	5.72	0.9992	88.51	14.3

[1]LOD—Limit of detection; [2]LOQ—Limit of quantification; [3]r^2—Correlation coefficient; [4]RSD—Precision in case of repeatability

Concentration of phthalates in honey

The pilot study results are presented in Table 4.

Table 4. Concentration of phthalates in honey and blank (μg/kg)

Compound	Blank (hexan + water)	3 years stored honey in glass jar[1]	3 years stored honey in plastic jar[2]
DMP	<LOQ	<LOQ	<LOQ
DEP	<LOQ	<LOQ	3.34
DiBP	<LOQ	5.32	15.84
DBP	<LOQ	1.32	16.01
DEHP	<LOQ	4.45	14.44
DnOP	<LOQ	< LOQ	< LOQ

[1] Average concentration of 5 measurements of 3 years stored honey in glass jar;
[2] Average concentration of 5 measurements of 3 years stored honey in plastic jar

DISCUSSION

Various analytical methods, liquid and gas chromatographic techniques with different detectors have been used to determine phthalates over the years. (Glaser et, 1981; Prokůpková et al, 2002; Batlle & Nerín, 2004; Li et al, 2004; Xu et al, 2007; Farajzadeh et al, 2012; Yan et al, 2012), LC–UV (Jen and Liu, 2006; Ling et al, 2007; Li et al, 2008; Zhao et al, 2008; Kamarei et al, 2011), GC–MS (Penalver et al, 2000; Feng et al 2005; Serôdio & Nogueira, 2006; Sørensen, 2006; Shen et al, 2007; Liu et al, 2008; Regueiro et al, 2008; Cacho et al, 2012; Jiao et al, 2012; Sun et al, 2012; Huang et al, 2012; Yan et al, 2012; Kong et al, 2012) and LC–MS/MS (López-Jiménez et al, 2005). GC–MS detection methods for the identification and quantification of phthalates are most commonly used for routine analyses due to their relatively high sensitivity and selectivity. In addition to the development of methods for quantifying phthalates, a significant step in this process is the development of a method for sample preparation. Over the years, different techniques and matrices have required different methods of sample preparation and optimization, in order to obtain a fast and reliable method of preparation. Besides SPE preparation (Harris et al, 1997; Liu et al, 2008; Li et al, 2008; Kamarei et al, 2011; Yan et al, 2012), single-drop microextraction (Batlle and Nerín, 2004), solid phase microextraction (Penalver et al, 2000; Prokůpková et al, 2002; Li et al, 2004; Feng et al, 2005), liquid- liquid extraction were also used (LLE) (Zhou et al, 2014).

In this study, performance development methods included verification of linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, recovery using a Guardiennes for pesticides residues (SANTE/2019). The calibration curves were obtained by measuring standard solutions injected in five level of concentration (0.005; 0.01; 0.05; 0.1; 0.5 μg/mL). Figure 1. shows chromatograms of solvent blank (water and hexane) and standard solution, which means that the applied parameters of the method will enable phthalates separation. The linearity of the method was determined by calibration in five calibration levels and good linearity characterized by a coefficient of linearity was obtained for all phthalates of interest > 0.999 ($r^2 > 0.999$). LOQ and LOD were determined by injecting five consecutive samples of the first calibration point (0.005 ug / mL) with an acceptable accuracy of ± 20%. The accuracy was assessed on the basis of the data obtained by injecting standard solutions in five replicates in two calibration levels. It ranged between 0.78 – 5.72. Recovery was determined by spiking a sample of honey into two concentration levels and two mean values obtained are shown as a result. Recovery values range between 88.51 - 112.23% which is in line with the Sante guidelines. The result of the recovery, which corresponds to the recommendation for residual

determinations, also shows us that the applied phthalate extraction method is satisfactory. During the development of the method, no calibration method was used through the matrix, but a blank sample was taken that underwent the same preparation procedure as the honey sample and contained water and hexane. The obtained results on the presence of phthalates in the blank are shown in Table 4. Namely, the values of phthalates in the blank are quantified, but below the LOQ values with a low level of confidence and therefore are not shown. However, it is important to point out that the solvent blank must work due to the wide distribution of phthalates. Notardonato et al (2020) got linearity > 0.999, but recovery was in the range from 69.3 to 98.8%. Zhou et al (2014) also got good linearity and recovery was between 82.9 – 110.9%.

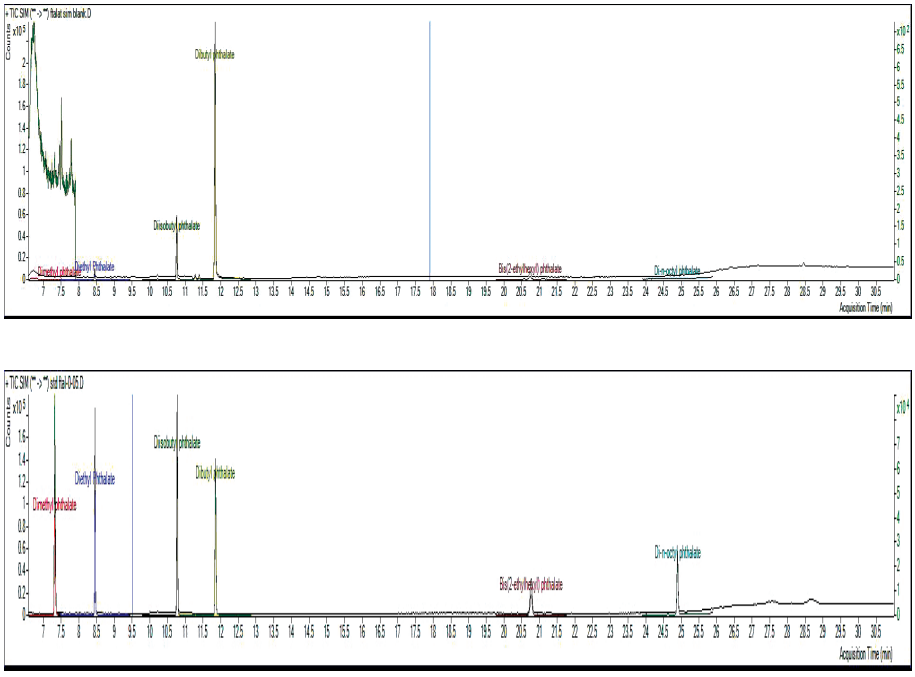

Figure 1. SIM mode blank chromatographs (hexane and water) (above) and phthalate standard with a concentration of 0.05 µg/g (below).

Notardonato et al (2020) also used gas chromatograph (GC) coupled with ion trap mass spectrometry (GC-MS) with selected ion monitoring (SIM) mode for phthalates determination same like Goodman W. (2009) who made an application note for determination of phthalates using GC-MS. If we look the condition parameters, we can see that we all had used similar parameters. We all used silica column with same diameters (30 m x 0.25 mm x 0.25 µm),

while the start temperature was not exactly the same, but it was similar (between 90 and 100 °C). However, the response for each compound was linear with r^2 greater than 0.999 for all phthalates compounds and for all authors.

In this study we analyzed and found phthalates in honey. Some other authors also reported phthalates contamination of honey (Notardonato et al, 2020; Zhou et al, 2014; Lo Turco et al, 2016). The concentration that we registered was much lower than that found by other authors who analyzed more samples (Notardonato et al., 2020; Lo Turco et al, 2016).

DMP and DnOP were not detected in these ten honey samples, while other analyzed phthalates were present. The maximum contents of PAEs were within 16.01 μg/kg for DBP; 15.84 μg/kg for DiBP; 14.44 μg/kg for DEHP; 3.34 μg/kg for DEP.

These examinations show that the samples in glass bottle had lover level of phthalates. Notardonato et al. (2020) analyzed 47 nectar honey samples and they found DMP in only one sample while DEP were found in 5 samples in the range from 25.4 to 374 μg/kg; DIPB were detected in six samples ranging between 28.7 and 553.1 μg/kg, DBP was in the range from 11.5 to 996.8 μg/kg; DEHP was detected in 37 samples in the range from 4.9 to 502.8 μg/kg and DnOP in range from 5.1 to 888.2 μg/kg in 25 samples. Our examination shows a lower level of contamination in honey than in other studies (Lo Turco et al, 2016; Notardonato et al., 2020;).

As some plastic can migrate from food through different materials, the EU Commission has defined the presence and the levels of small amounts of additives in food. Particularly, according to the EU Regulation No. 10/2011 and 2005/2018, the safety limit defined by each specific migration limits (SMLs) in food, for DMP, DEP, DiBP, and DnOP is 60 mg/kg, and 0.3, 0.05, and 1.5 mg/kg for DBP, BP-A, and DEHP, respectively. The limit of 60 mg/kg requires consideration: this high value means that the additive is permitted to be used in the polymer production for food packaging and there are no restrictions. The LOQs that we had were lower than the SML set by the EU Commission: this means that the method investigated is sensitive enough to analyze the threshold limits of the different compounds in the collected honey samples.

CONCLUSION

In this study, a fast and reliable method for the determination of six phthalates from honey was developed. Using liquid-liquid extraction, satisfactory recovery values were obtained and the analysis was performed on GC-MS. This paper can be considered as a pilot study for the determination of phthalates in honey and honey products. During the analysis, phthalates were found even

in blank solvent, in concentrations lower than LOQ, and their presence even in the blank indicates the need to expand the study. During the test, phthalates were found in all tested honey samples. Given the findings, in the future, we will focus on potential sources of phthalate contamination in the process of production of honey and honey products and will continue more extensive testing of these contaminants not only in honey but also in other foods.

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Author's Contribution

B.K. drafting the manuscript and have made substantial contributions to basic idea; B.K. and J.P. carried out the GC-MS analysis and have been involved in drafting the manuscript; J.V. carried out sample collection and sample preparation and performed the statistical analysis; J.V. and R.R. have been involved in drafting the manuscript; B.Đ. have been involved in data collection; J.V. and R.R. revised the manuscript critically.

Competing interest

The authors declare that they have no competing interests.

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ANTIMICROBIAL DRUGS, PESTICIDES AND PAHs IN HONEY

Jelena Petrović[1*], Radomir Ratajac[1], Jelena Vranešević[1], Igor Stojanov[1], Jasna Prodanov Radulović[1], Brankica Kartalović[1]

[1]Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia

Abstract

Honey is a very popular food that is often consumed by immunocompromised people, so it is crucial that it is safe. The safety of honey is related to numerous hazards, especially the chemical ones like residues of sulfonamides, lindane and polycyclic aromatic hydrocarbons (PAHs). Honey from our country contains high concentrations of banned antimicrobial substances such as sulfonamides (3.9% of samples). The frequent presence of lindane in honey is not unexpected considering the fact that this pesticide is present in the soil, plants and animals from Serbia. PAHs as a relatively unexplored chemical hazard in honey proved to be widespread in the honey from Serbia - even 6.6% of honey samples contains toxic concentrations of these compounds. The control of chemical hazards and the production of safe honey in Serbia could be improved by implementing better measures to prevent illegal use of antimicrobial drugs, pollution control and further research in the field of risk assessment.

Key words: honey, sulfonamides, lindane, PAHs, food safety

[1*] Corresponding Author: jelena@niv.ns.ac.rs

ANTIMIKROBNI LEKOVI, PESTICIDI I PAH U MEDU

Jelena Petrović[1*], Radomir Ratajac[1], Jelena Vranešević[1],
Igor Stojanov[1], Jasna Prodanov Radulović[1], Brankica Kartalović[1]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija

Kratak sadržaj

Med je vrlo popularna namirnica koju često konzumiraju imunokompromitovane osobe, stoga je njegova bezbednost od izuzetnog značaja. Bezbednost meda je određena brojnim hazardima među hemijskim se ističu rezidue sulfonamida, lindana i policikličnih aromatičnih ugljovodonika (PAH). Med sa prostora naše zemlje sadrži visoke koncentracije zabranjenih supstanci kao što su sulfonamidi (3.9% uzoraka). Često prisustvo lindana u medu nije neočekivano s obzirom da je ovaj perzistentni pesticid prisutan u zemljištu, biljkama i životinjama Srbije. Policiklični aromatični ugljovodonici kao relativno ne proučavani hemijski hazardi meda su se pokazali kao vrlo prisutni u medu iz Srbije, čak 6.6% uzoraka sadrži toksične koncentracije ovih jedinjenja. Unapređenje kontrole hemijskih hazarda i proizvodnja bezbednog meda u Srbiji bi se postigli efikasnijom primenom mera za sprečavanje ilegalne upotrebe antimikrobnih lekova, kontrolom zagađenja i daljim istraživanjima u oblasti procene rizika.

Ključne reči: med, sulfonamidi, lindan, PAH, bezbednost hrane

INTRODUCTION

Honey is the most important product of honey bees. It has been used in the diet since prehistoric times, mainly because of its pleasant taste and nutritional properties. Honey is often consumed by immunocompromised people because it's medical effect. Honey has antioxidant, bioactive, antimicrobial, anti-inflammatory, antithrombotic and antiallergic effects, depending on the type (Cook and Sammon, 1996). Since honey is often consumed by vulnerable population, the standards set for its safety are very strict (Wei et al., 2012). Hazards, i.e. dangers to the health of consumers, which are most commonly related to the safety of honey, are residues of veterinary medical products and pesticides. In our recent research, it has been found that there is a risk associated with the presence of polycyclic aromatic hydrocarbons (PAHs) in honey.

Bees collect nectar in a radius of 3 to 10 km around the hives, so bees, pollen and honey are considered bioindicators of environmental pollution, especially when it comes to radionuclides, PAHs and pesticides. (Bogdanov 2006; Babić et al., 2017; Petrović et al., 2019).

When assessing the importance of certain chemical hazards for the safety of honey, it is necessary to keep in mind the fact that honey differs greatly from other foods of animal origin. When some of the chemical hazards enter the body of mammals or birds, it undergoes active metabolism, where chemical substances are transformed into metabolites which are then deposited in the tissues of the animal and more or less excreted from the body through urine, feces, milk, etc. Chemical hazards usually reach the nectar or sugar syrup that bees feed on. Bees then secrete enzymes that break down the nectar into simple sugars: glucose, fructose and sucrose and deposit the nectar in the honeycombs, and over time the water evaporates and thick syrup is formed - honey (Solomon et al., 2006). If veterinary drugs, pesticides, PAHs are present in nectar or sugar syrup, they are transported in unchanged form in honey (Reybroeck et al., 2012). Decomposition of labile chemical compounds can occur in honey. However, sulfonamides, PAHs and lindane are very stable, so not only does the concentration not decrease, but due to water evaporation, the initial concentration increases, which does not change until the end of honey storage. Therefore, once contaminated honey remains contaminated over a long period of time and can pose a risk to the health of consumers, particularly immunocompromised population.

The main goal of this paper is to present the results of our research on the presence of sulfonamide residues, lindane and PAHs in honey produced in our country and to point out the importance of these chemical hazards for the safety of our honey.

SULFONAMIDES

In the past, the use of sulfonamides was allowed in beekeeping for the treatment of American bee brood plague (Plavša et al. 2008). However, the appearance of residues of these drugs in honey produced by treated bees has led to a ban on the use of sulfonamides in the treatment of bee diseases in the USA (Barganska et al, 2011). If food with sulfonamide residues is consumed, allergic reactions may occur (Maroubo et al., 2021). In our country, like in most EU countries, it is not allowed to treat bees with antimicrobial drugs (Kartalović et al., 2020). Therefore, any occurrence of sulfonamide residues in honey is a result of their intentional and illegal use for prevention and treatment of bee diseases.

Examination of honey samples from Serbia revealed the presence of sulfonamides in 3.9% of samples (Table 1). The residues of sulfadiazine (3.9%) were most commonly found, followed by sulfamethoxazole (1.7%) and sulphapyridine (1.1%). Illegal substances are common in flower 28.6%, sunflower 10.0%, meadow 3.8% and acacia honey (1.7%) (Kartalović et al., 2020). Due to the ban on the use of sulfonamides in beekeeping, all the samples shown in Table 1 are not safe for human consumption. Three honey samples (sunflower, meadow and acacia) had extremely high total content of sulfonamide residues - it was even higher than the maximum residue limits (MRL) of sulfonamide residues for any food in the EU.

Table 1: Honey samples with quantified residue (ng/g) (Kartalović et al., 2020)

sample	sulfadiazine	sulfamethizole	sulfopyridine	sum
sunflower	11	370	< LOQ	381
meadow	< LOQ	59	< LOQ	59
meadow	7.8	< LOQ	< LOQ	7.8
meadow	97	< LOQ	9.1	106.1
flower	9.2	< LOQ	8.2	17.4
acacia	< LOQ	208	< LOQ	208
average	31.3	212.3	8.7	129.9
sd	43.9	155.5	0.6	143
min	7.8	59	8.2	7.8
max	97	370	9.1	381

LOQ limit of quantification; sd - standard deviation

Sulfonamides are very stable in honey. One year after the ending of treatment of bees, the honey they produce contains sulfonamides in the amount of 1000 ng/g (Reybroeck et al., 2010). Furthermore, due to their stability, sulfonamides are considered environmental pollutants (Chen and Xie, 2018). Therefore, once contaminated, honey remains an unsafe food until the end of its shelf life.

Examination of honey in the EU imported from third countries showed that 20 - 50% of the samples contained residues of antimicrobial drugs, most commonly streptomycin, sulfonamides, tetracyclines and chloramphenicol. However, honey produced in the EU also contained residues of antimicrobial drugs in 1 - 7% of the examined samples (Bogdanov, 2006). The results of our research indicate that the contamination of honey residues in Serbia is at the level of the European average.

In our research it was found that the average concentration of sulfonamides is 10 times higher in honey compared to meat produced in Serbia, and the content of residues in meat is not higher than the maximum residue limits (Kartalović et al, 2020). In farm breeding of cattle, pigs and broilers, sulfonamides are registered drugs and their usage (doses, length of application) is precisely prescribed, so it is possible to meet the withdrawal period deadlines and as a final result the meat that reaches the market is safe in terms of sulfonamide residues. However, sulfonamides are not allowed in beekeeping, so beekeepers use them arbitrarily, probably in very high doses, and as a final outcome, 3.9% of honey on our market is unsafe only due to the presence of sulfonamides.

PESTICIDES

Pesticides that can be found in honey usually belong to the group of insecticides used against varroosis (amitraz, coumaphos, etc.). These preparations are applied directly in the hive. However, pesticides that are persistent organic pollutants (POPs) can get into honey as well. Lindane belongs to the group of POPs. It was once used as an insecticide. In addition to the active component, commercial lindane preparations always contain other isomers, mainly α, β and δ. After application, lindane mainly accumulates in the soil, and that is how it can reach groundwater and herbs. Due to volatilization and wind erosion, lindane is easily transmitted by air and can reach long distances. Lindane is very stable in the environment (Sandu and Vrista, 2015). Due to its bioaccumulation and toxic effects on human health, lindane has been banned in many countries for decades, but it can still be found in human blood and soil (Mohapatra and Pandey, 2015). In the territory of Vojvodina, lindane was found in soil, wheat and animal tissues (Škrbić 2007; Petrović et al., 2021) but also in imported food (Kartalović et al, 2016). Lindane is often found in wild pigs in Vojvodina (64.6%), which indicates persistent contamination of the environment and wild animals (Petrović et al., 2021). Lindane reaches honey as a result of environmental contamination. The concentrations in honey are lower than the concentrations found in bees or pollen (Fléché et al. 1997; Schur and Wallner, 2000).

In our research, the average concentration of lindane was determined to be in the range 3 - 5 ng/g of acacia, meadow and sunflower honey, while in linden and forest honey the average concentration was lower than 0.5 ng/g. Lindane makes up for 97% of the total organochlorine pesticide content in acacia honey, 85% in linden honey, 73% in meadow and sunflower honey and 58% in forest honey (Kartalović et al, 2015). In African and Asian countries, the level of lindane in honey is far higher - 14.4 ng/g Uganda (Mukibii et al.,

2021), 26.9 ng/g in Pakistan (Rafique et al., 2018), while the content in honey from Serbia is far more similar to the average content in European countries: 3.7 ng/g in Turkey, 3.87 ng/g in Italy, 8.5 ng/g in France (Chauzat et al. 2009; Yavuz et al., 2010; Saitta et al., 2017). The differences in the content are caused by the ban on the use of lindane from different periods. China banned lindane only in 2019 (Mukibii et al., 2021).

Toxicological analyses have shown that consuming honey with a high content of organochlorine pesticides, especially lindane, dieldrin, DDT and endosulfan, can lead to reproductive toxicity: reduced sperm quality, low testosterone levels, reduced testicular weight, abortion (El-Nahhal, 2020). The content of pesticides in honey that can harm human health has been proven in Turkey, Italy (Yavuz et al., 2010; Saitta et al., 2017), India, Pakistan (Rafique et al., 2018), Uganda (Mukibii et al., 2021).

POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

PAHs are organic compounds formed during the pyrolysis process (fires, industrial pollution, heating, etc.) (EFSA 2008; Purcaro et al. 2013). PAHs can reach foods during food processing (smoking, grilling) or as a result of environmental pollution (Singh et al. 2016; Mastanjević et al., 2019). Since PAHs have mutagenic and carcinogenic properties, ML (Maximum limits) are prescribed for different types of food. Our legislation is the same as in the EU (Petrović et al., 2019).

PAHs can get into honey in two ways from the environment or during the smoking of hives. PAHs are lipophilic and they most commonly accumulate in a bee itself as they are directly exposed to contamination, they bind more to pollen than to nectar, while the lowest concentrations are in honey (Lambert et al., 2012). During their work, beekeepers often smoke hives and the procedure might be a source of PAHs even though they are found in honey before the hives are smoked (Lambert et al., 2012). A possible source of contamination is an inadequate use of smoke sticks used for treatment against *Varroa*.

In our research, the presence of all 16 PAHs from the EPA list as hazardous substances was determined in honey (Petrović et al, 2019). Results are presented in Table 2. The average content of 16 PAHs ranged from not detected to 2.35 ng/g, and the highest average concentrations were determined for anthracene. The presence of phenanthrene and anthracene was detected in 90.16 - 95.08% of the tested samples at concentrations of 1 - 6 ng/g. Carcinogenic potential of food is determined by the sum of PAH8 (benzo[a]pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi] perylene, dibenz[a,h]anthracene, indeno[1,2,3-cd]pyrene) or by an individual

finding of some of them. In our honey tests, 72.1% of honey samples did not have carcinogenic and genotoxic potential, since PAH8 < 1 μg/kg was measured in them. In other samples (27.9%) risky amounts of PAH8 were detected and in four samples of polyfloral honey (6.6%) extreme values of PAH8 were detected (58.9 -656.2 ng/g PAH8).

Table 2: PAH16 in honey samples (Petrović et al., 2019)

PAH	Meadow n=34	Sunflower n=4	Accacia n=19	Forest n=4	Total n=61
Naphtalene					
< LOD[1] (%)	17 (50.00)	4 (100.00)	5 (26.32)	2 (50.00)	28 (45.90)
Min - Max μg/kg	0.00 - 1.25	0.05 - 0.29	0.06 - 1.08	0.15 - 0.90	0.00 - 1.25
Median ± SD μg/kg	0.35 ± 0.40	0.13 ± 0.11	0.64 ± 0.26	0.43 ± 0.32	0.48 ± 0.35
Acenaphthylene					
< LOD (%)	26 (76.47)	3 (75.00)	16 (84.21)	2 (50.00)	47 (78.33)
Min - Max μg/kg	0.00 - 1.59	0.10 - 2.85	0.04 - 4.74	0.14 - 6.24	0.00 - 6.24
Median ± SD μg/kg	0.27 ± 0.30	0.23 ± 1.34	0.15 ± 1.07	2.83 ± 3.29	0.23 ± 1.21
Acenaphthene					
< LOD (%)	14 (41.18)	1 (25.00)	8 (42.11)	1 (25.00)	24 (40.00)
Min - Max μg/kg	0.00 - 3.39	0.17 - **15.38**	0.09 - 1.15	0.32 - 0.94	0.00 - **15.38**
Median ± SD μg/kg	0.49 ± 0.71	0.68 ± 7.44	0.39 ± 0.31	0.81 ± 0.28	0.49 ± 1.98
Fluorene					
< LOD (%)	13 (38.24)	2 (50.00)	6 (31.58)	1 (25.00)	22 (36.67)
Min - Max μg/kg	0.00 - 0.93	0.05 - 0.77	0.03 - 3.22	0.35 - 2.31	0.00 - 3.22
Median ± SD μg/kg	0.44 ± 0.27	0.53 ± 0.33	0.45 ± 0.69	0.56 ± 0.91	0.45 ± 0.50
Phenanthrene					
< LOD (%)	5 (14.71)	1 (25.00)	0	0	6 (10.00)
Min - Max μg/kg	0.00 - 3.70	0.28 - 2.14	1.37 - 3.10	1.39 - 2.43	0.00 - 3.70
Median ± SD μg/kg	1.97 ± 0.91	1.61 ± 0.83	1.91 ± 0.52	1.83 ± 0.55	1.93 ± 0.78
Anthracene					
< LOD (%)	2 (5.88)	1 (25.00)	0	0	3 (5.00)
Min - Max μg/kg	0.00 - 6.51	0.22 - 3.57	1.68 - 3.42	1.69 - 2.96	0.00 - 6.51
Median ± SD μg/kg	2.38 ± 1.20	2.11 ± 1.43	2.33 ± 0.58	2.24 ± 0.67	2.35 ± 1.02
Fluoranthene					
< LOD (%)	31 (91.18)	4 (100.00)	19(100.00)	4 (100.00)	58 (96.67)
Min - Max μg/kg	0.00 - 3.61	0.19 - 0.25	0.01 - 0.34	0.09 - 0.23	0.00 - 3.61
Median ± SD μg/kg	0.19 ± 0.60	0.24 ± 0.03	0.17 ± 0.09	0.17 ± 0.06	0.19 ± 0.45

PAH	Meadow n=34	Sunflower n=4	Accacia n=19	Forest n=4	Total n=61
Pyrene					
< LOD (%)	31 (91.18)	4 (100.00)	19(100.00)	4 (100.00)	58 (96.67)
Min - Max µg/kg	0.00 - 3.61	0.19 - 0.25	0.01 - 0.34	0.09 - 0.23	0.00 - 3.61
Median ± SD µg/kg	0.19 ± 0.60	0.24 ± 0.03	0.17 ± 0.09	0.17 ± 0.06	0.19 ± 0.45
Benz[*a*]anthracene					
< LOD (%)	29 (85.29)	4 (100.00)	19 (100.00)	4 (100.00)	56 (93.33)
Min - Max µg/kg	0.00 - **87.24**	0.19 - 0.25	0.00 - 0.12	0.00 - 0.06	0.00 - **87.24**
Median ± SD µg/kg	0.00 ± 15.16	0.24 ± 0.03	0.00 ± 0.04	0.00 ± 0.03	0.00 ± 11.39
Chrysene					
< LOD (%)	29 (85.29)	4 (100.00)	18 (94.74)	4 (100.00)	55 (91.67)
Min - Max µg/kg	0.0 - **140.58**	0.00 - 0.00	0.00 - 1.51	0.00 - 0.32	0.0 - **140.58**
Median ± SD µg/kg	0.00 ± 24.38	0.00 ± 0.00	0.00 ± 0.39	0.02 ± 0.16	0.00 ± 18.30
Benzo[*b*] fluoranthene					
< LOD (%)	29 (85.29)	4 (100.00)	19 (100.00)	4 (100.00)	56 (93.33)
Min - Max µg/kg	0.00 - **23.91**	0.00 - 0.00	0.01 - 1.99	0.02 - 0.18	0.00 - **23.1**
Median ± SD µg/kg	0.11 ± 4.96	0.00 ± 0.00	0.07 ± 0.44	0.13 ± 0.07	0.19 ± 0.45
Benzo[*k*] fluoranthene					
< LOD (%)	29 (85.29)	4 (100.00)	19 (100.00)	4 (100.00)	56 (93.33)
Min - Max µg/kg	0.00 - **79.65**	0.05 - 0.13	0.00 - 2.43	0.01 - 0.18	0.00 - **79.65**
Median ± SD µg/kg	0.11 ± 13.85	0.07 ± 0.03	0.06 ± 0.54	0.14 ± 0.08	0.10 ± 10.41
Benzo[a]pyrene					
< LOD (%)	26 (76.47)	4 (100.00)	19 (100.00)	4 (100.00)	53 (88.33)
Min - Max µg/kg	0.0 - **120.15**	0.05 - 0.13	0.00 - 0.12	0.00 - 0.78	0.0 - **120.15**
Median ± SD µg/kg	0.09 ± 20.89	0.07 ± 0.03	0.00 ± 0.03	0.04 ± 0.38	0.04 ± 15.69
Indeno[*cd*]pyrene					
< LOD (%)	28 (82.35)	4 (100.00)	18 (94.74)	4 (100.00)	54 (90.00)
Min - Max µg/kg	0.00 - **38.68**	0.00 - 0.04	0.01 - 4.85	0.03 - 0.23	0.00 - **38.68**
Median ± SD µg/kg	0.07 ± 8.22	0.00 ± 0.02	0.03 ± 1.10	0.06 ± 0.09	0.06 ± 6.27
Dibenz[*a,h*] anthracene					
< LOD (%)	28 (82.35)	4 (100.00)	18 (94.74)	4 (100.00)	54 (90.00)
Min - Max µg/kg	0.00 - **39.15**	0.01 - 0.27	0.00 - 1.86	0.01 - 0.24	0.00 - **39.15**
Median ± SD µg/kg	0.06 ± 7.36	0.05 ± 0.12	0.03 ± 0.42	0.05 ± 0.10	0.05 ± 5.57

PAH	Meadow n=34	Sunflower n=4	Accacia n=19	Forest n=4	Total n=61
Benzo[*ghi*] perylene					
< LOD (%)	29 (85.29)	4 (100.00)	18 (94.74)	4 (100.00)	55 (91.67)
Min - Max µg/kg	0.0 - **136.34**	0.01 - 0.27	0.00 - 3.65	0.00 - 0.01	0.0 - **136.34**
Median ± SD µg/kg	0.02 ± 23.56	0.24 ± 0.12	0.00 ± 0.83	0.00 ± 0.01	0.02 ± 17.68

[1] Limit of the detection

Our research shows that there is a certain risk related to the content of PAHs in honey. It was not possible to define a common PAHs profile for the tested honey samples, which means that there are different sources of contamination. However, the impact of some beekeeping procedures such as smoking and the use of drugs against *Varroa* have not yet been sufficiently studied and is the subject of future research along with risk assessment for PAHs in honey.

CONCLUSION

Honey is considered both food and medicine and it is often consumed by the sick and children, and therefore it needs to be absolutely safe for consumption. Our research has confirmed that certain risky substances can be found in honey originating from Serbia. Persistent contaminants such as lindane cannot be eliminated from the environment but further toxicological studies are needed in order to determine whether risky amounts of this pesticide are consumed in the average quantities that people normally take. The content of PAHs that reach honey as a result of environmental pollution (cars, heating, etc.) can be controlled, while further research should determine whether the common methods of hive smoking leave toxic content in honey. And finally, the presence of sulfonamides as a direct result of illegal and irresponsible work of beekeepers poses a significant risk for honey consumption in Serbia. The risk for consumers can be eliminated by educating beekeepers and by introducing strict measures in the trade of antimicrobial drugs.

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Author's Contribution

This paper was written as a review paper which dealt with a series of papers in the field of honey safety. All authors of this paper have participated in the writing of this paper and previous research. Writing papers JP and JPR, examinations BK and JV, and data processing RR and IS.

Competing interest

The authors declare that they have no competing interests.

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RADIOACTIVE RESIDUE IN HONEY

Željko Mihaljev[1], Sandra Jakšić[1], Milica Živkov Baloš[1*], Nenad Popov[1]

[1]Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia

Abstract

The concentration of radioactive isotopes in honey is an important bioindicator of environmental contamination. For that purpose, a total of 66 samples of different types of honey (acacia, meadow, linden, sunflower, flower, oilseed rape, chestnut) were examined. The samples were collected during 2020 and 2021 at different localities in the Republic of Serbia (Vojvodina, Central Serbia, Kosovo and Metohija). Gamma spectrometric analysis was used to determine natural radionuclides potassium-40 (^{40}K), thorium-232 (^{232}Th), radium-226 (^{226}Ra), uranium-238 (^{238}U), uranium-235 (^{235}U) and sodium-22 (^{22}Na) and anthropogenic radionuclide caesium-137 (^{137}Cs). The obtained results indicate that the predominant radionuclide in all the analyzed honey samples is natural K-40, whose average activity was 74 Bq/kg. The activity of other tested radionuclides ranged as follows: Th-232: < 1 - 2.0; Ra-226: 1.9 - 15.6; U-238: < 1 - 31.4; U-235: < 0.2 - 1.61 and Na-22: < 0.2 - 2.4 Bq/kg. The activity of the artificial radionuclide Cs-137 was measured in 53% of the tested samples from the territory of Kosovo with the maximum value of 3.63 Bq/kg. Regarding the determined level of radioactive residues, it can be concluded that the honey produced in the Republic of Serbia is healthy and environmentally safe food.

Key words: honey, natural radionuclides, artificial radionuclides

[1*] Corresponding Author: milica@niv.ns.ac.rs

RADIOAKTIVNE REZIDUE U MEDU

Željko Mihaljev[1], Sandra Jakšić[1], Milica Živkov Baloš[1*], Nenad Popov[1]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija

Kratak sadržaj

Koncentracija radioaktivnih izotopa u medu predstavlja važan bioindikator kontaminacije životne sredine. U tom cilju ispitano je ukupno 66 uzoraka različitih vrsta medova (bagremov, livadski, lipov, suncokretov, cvetni, kestenov i od uljane repice,). Uzorci su sakupljeni u toku 2020/2021 godine sa različitih lokaliteta Republike Srbije (Vojvodina, centralna Srbija, Kosovo i Metohija). Gama-spektrometrijskom analizom određivani su prirodni radionuklidi kalijum-40 (^{40}K), torijum-232 (^{232}Th), radijum-226 (^{226}Ra), uran-238 (^{238}U), uran-235 (^{235}U) i natrijum-22 (^{22}Na) kao i antropogeni radionuklid cezijum-137 (^{137}Cs). Dobijeni rezultati ukazuju da je predominantni radionuklid u svim analiziranim uzorcima meda prirodni K-40, čija je prosečna aktivnost iznosila 74 Bq/kg. Aktivnost ostalih ispitanih radionuklida kretala se u intervalu za Th-232: < 1 - 2.0; za Ra-226: 1.9 - 15.6; za U-238: < 1 - 31.4; za U-235: < 0.2 - 1.61 i za Na-22: < 0.2 - 2.4 Bq/kg. Aktivnost veštačkog radionuklida Cs-137 izmerena je u 53% ispitanih uzoraka sa područja Kosova sa maksimalnom vrednošću od 3.63 Bq/kg. S obzirom na utvrđeni nivo radioaktivnih rezidua može se zaključiti da je med proizveden u Republici Srbiji zdrava i ekološki bezbedna hrana.

Ključne reči: med, prirodni radionuklidi, veštački radionuklidi

INTRODUCTION

Rapid development of industry and technology worldwide is leading to an increase in global environmental pollution. This can result in the presence of a large number of contaminants of various origins in foods for human consumption, with radionuclides being the most significant ones. They are primarily the result of the application of nuclear energy for peacetime use. Past accidents at nuclear power plants (Chernobyl, 1986; Fukushima, 2011) have caused high levels of radioactive contamination of the biosphere. Increased content of radionuclides in agricultural land as a result of the use of artificial phosphate fertilizers and coal combustion in thermal power plants with their solid waste (ash, slag) are the most important source of “technologically increased natural

radioactivity" because they contain natural radionuclides (uranium, thorium) and their derivatives. Also, during the bombing in 1999, the territory of the Republic of Serbia was contaminated with depleted uranium and the consequences of this are long-term and unforeseeable. All these factors indicate that it is significant to measure the level of radioactivity in the environment and predict the effects it might have for the flora and fauna, humans and ecosystems in different ways in the years to come (Blagojević and Simić, 2012). These sources of radioactive contamination provide ample opportunities for disturbing the ecological balance because they are by far the largest anthropogenic source of increasing the content of radionuclides in the soil, and therefore also in plants and other links in the food chain. Honey bee (*Apis mellifera*) has an extremely important and complex role in nature because it affects the production of human and animal food, various industrial raw materials of agricultural origin as well as human health. However, the conditions for beekeeping have been deteriorating for a long time because plants absorb various harmful substances like radionuclides, through roots, leaves and flowers - and the flowers of plants are the main sources of bee food, nectar and pollen. It has also been documented (Mihaljev et al., 1992) that honey contains chemical components of plants. Honey bees collect pollen and nectar from the environment, so the content of elements and their amount in honey depends on environmental conditions. Honey is a food that is exclusively a product of honey bees. Its quality depends on the type and geographical origin, environmental conditions, the origin of bees, the method of processing and storage. It is not allowed to change the composition of honey by adding some substances.

Bees are a part of nature and represent one of the most nutritious foods, which is rich in many nutrients of great physiological and preventive value for the human body. However, bees are also exposed to radioactive substances, i.e., ionizing radiation that is in the environment. Some observations after the Chernobyl nuclear disaster showed that in some areas bees had very intense reaction to increased radioactivity like anxiety, disorientation and mass abandonment of hives (Vorgić, 2004). Increased content of radionuclides on bee pastures can affect general health of bees, and the presence of radionuclides in honey can significantly change the quality of this high-quality food. Given the characteristics of honey (Stanimirović et al., 2000), which imply a relatively wide area where bees collect nectar, honey is certainly one of the potential bioindicators of radioactive contamination.

MATERIAL AND METHODS

A total of 66 honey samples (acacia, meadow, linden, sunflower, flower, oilseed rape, chestnut) were collected from 2020 to 2021. The specific activities of caesium-137 (^{137}Cs), potassium-40 (^{40}K), uranium-238 (^{238}U), uranium-235 (^{235}U), radium-226 (^{226}Ra), thorium-232 (^{232}Th) and sodium-22 (^{22}Na) were determined using a coaxial HPGe detector (ORTEC-AMETEK, GEM25P4-70) with an energy resolution of 1.67 keV at the 1.33 MeV of ^{60}Co, Peak-to-Compton ratio 67:1 (Amp Shape Time 6μs) and the relative efficiency of 28%. The detector was housed in a 10 cm thick lead shield lined by copper layer, cadmium layer and a layer of plexiglass (IAEA, 1989). The instrument was calibrated by the accredited laboratory for gamma-ray spectroscopy calibration and activity of radioactive sources of gamma emitters - Chair of Nuclear Physics, Department of Nuclear Physics, Faculty of Sciences, University of Novi Sad. The precision and accuracy of the instrument operation was controlled using standard reference material Source No. LR 320 certified by the Calibration Laboratory for Measurements of Radioactivity (Deutscsher kalibrierdienst, Braunschweig, Germany).

The counting time for each sample was 100,000 s, and the analytical precision of the measurement was approximately ± 10%. The specific activity of ^{238}U was calculated from gamma-rays of ^{234}Th at 63.3 keV and gamma-rays Pa-234m (1001keV). The specific activity of ^{226}Ra was determined from the peak areas at 609.3, 1120.3 and 1764.5 keV of ^{214}Bi and 295.2 and 351.9 keV of ^{214}Pb. Gamma-ray peaks with energies 911.2 and 969.0 keV (^{228}Ac) and gamma-ray doublet 238.6 keV (^{212}Pb) and 241 KeV (^{214}Pb) as well as Tl-208 (583.2 KeV) were used for calculation of the specific activities of ^{232}Th. Specific activities of ^{40}K and ^{137}Cs were determined using their own 1460.8 keV and 661.7 keV peaks, respectively (Bikit et al., 1995).

Input signals to the detector through ORTEC type pre-amplifiers and spectra amplifiers were channelled to a multichannel analyser MCA with an analog-to-digital converter of 16384 channels total memory. MCA was directly connected with PC where the measured spectra were stored and analysed. The gamma spectra were acquired and analysed using the GammaVision® software. This program calculates the activity concentration of an isotope from all prominent gamma lines after background subtraction. All the measurement of uncertainties are presented at 95% confidence level. The measured specific activity of ^{137}Cs was decay corrected to the sampling date (Ortec, 2015).

Results

The content of radioactive substances - radionuclides on honey plants is the result of the transfer of radioactive fallout from the atmosphere through air currents, water (rain, snow, erosive processes) and soil, or their direct and indirect reach to crops of meadows, pastures and forests. Gamma-spectrometric analysis in the examined samples of honey determined the presence of natural radionuclides, namely: sodium-22, potassium-40, thorium-232, radium-226, uranium-238 and uranium-235. The results of the measured activities of these radionuclides are shown in Table 1 and Table 2.

Table 1. The results of measurements of radionuclides in honey from the area of Vojvodina and Central Serbia

Radionuclides	**Cs-137**	**K-40**	**Th-232**	**Ra-226**	**U-238**	**U-235**	**Na-22**
Type of honey (number of samples)	**Activity concentration [Bq/kg]**						
1. Meadow honey (n=11)	< 0.5	54 ± 4	< 2	1.9 ± 1.0	< 10	< 1	2.2 ± 1.0
2. Acacia (n=9)	< 0.5	55 ± 6	< 1	< 1	< 10	< 1	< 1
3. Flower (n=8)	< 0.5	34 ± 2	1.8 ± 0.6	4.7 ± 1.1	14.3 ± 3	0.73 ± 0.15	1.8 ± 1
4. Sunflower (n=10)	< 0.5	32 ± 2	< 1	3.2 ± 1.0	16.6 ± 4.0	0.85 ± 0.05	2.2 ± 0.5
5. Linden (n=4)	< 0.5	41 ± 3	2.0 ± 0.7	6.6 ± 1.4	< 10	< 1	< 1
6. Oilseed rape (n=6)	< 0.5	74 ± 2	< 0.5	11.1 ± 1.8	21.5 ± 5.1	1.0 ± 0.01	2.2 ± 1.0
7. Chestnut honey (n=3)	< 0.5	74 ± 2	1.0 ± 0.5	4.2 ± 1.2	15.9 ± 1.7	0.82 ± 0.09	2.0 ± 1.0
Variation interval	**< 0.5**	**32 - 74**	**< 1 – 2.0**	**1.9 – 11.1**	**< 10–21.5**	**< 1 – 0.82**	**< 1 – 2.2**

When it comes to artificial (anthropogenic) radionuclides, the activity of cesium-137 was recorded, but only in the honey samples originating from the area of Kosovo and Metohija (Table 2). A total of 15 samples from different

localities were tested, and the presence of biologically significant ^{137}Cs radionuclide was determined at eight localities (which represents more than 50% of the total tested samples). It is important to note that all the measured activities of the isotope cesium-137 in the analysed honey samples are below the maximum allowable values (Official Gazette of RS, No. 36/2018).

Table 2. The results of measurements of radionuclides in honey from the area of Kosovo and Metohija

Radio-nuclides	**Cs-137**	**K-40**	**Th-232**	**Ra-226**	**U-238**	**U-235**	**Na-22**
Locality	**Activity concentration [Bq/kg]**						
1.	1.62 ± 0.19	88 ± 3	0.82 ± 0.10	6.14 ± 0.38	< 1	< 0.2	< 0.5
2.	0.41 ± 0.12	77 ± 3	1.04 ± 0.10	6.53 ± 0.35	< 1	< 0.2	1.37 ± 0.09
3.	< 0.7	123 ± 5	1.64 ± 0.20	10.4 ± 1.0	< 1	< 0.2	< 0.5
4.	< 0.6	100 ± 6	1.15 ± 0.21	4.67 ± 0.53	< 1	< 0.2	1.59 ± 0.17
5.	1.43 ± 0.25	68 ± 4	0.99 ± 0.20	2.44 ± 0.48	< 1	< 0.2	1.44 ± 0.16
6.	0.42 ± 0.11	70 ± 4	0.83 ± 0.11	6.80 ± 0.33	13.8 ± 3.7	0.71 ± 0.19	1.43 ± 0.08
7.	0.52 ± 0.06	106 ± 2	0.84 ± 0.06	11.1 ± 0.3	12.5 ± 2.1	0.64 ± 0.11	1.46 ± 0.06
8.	1.85 ± 0.18	103 ± 3	0.99 ± 0.13	4.35 ± 0.33	13.6 ± 3.5	0.70 ± 0.18	1.59 ± 0.09
9.	< 0.6	96 ± 4	1.67 ± 0.19	24.8 ± 0.9	< 1	< 0.2	2.94 ± 0.16
10.	< 0.4	43 ± 2	1.11 ± 0.10	7.52 ± 0.33	< 1	< 0.2	< 0.5
11.	< 0.3	34 ± 2	0.90 ± 0.08	9.20 ± 0.31	25.8 ± 5.4	1.32 ± 0.28	1.53 ± 0.07
12.	< 0.2	35 ± 2	0.88 ± 0.10	4.52 ± 0.32	31.4 ± 7.6	1.61 ± 0.39	1.42 ± 0.09
13.	< 0.3	112 ± 4	1.09 ± 0.11	15.6 ± 0.42	< 1	< 0.2	1.64 ± 0.10
14.	3.63 ± 0.31	107 ± 5	1.01 ± 0.20	4.06 ± 0.48	< 1	< 0.2	1.72 ± 0.14
15.	1.61 ± 0.29	94 ± 6	1.96 ± 0.38	7.34 ± 0.67	< 1	< 0.2	2.40 ± 0.29
Variation interval	**< 0.2 - 3.63**	**34 - 123**	**0.82 - 1.67**	**2.44 - 15.6**	**< 1 - 31.4**	**< 0.2 - 1.61**	**< 0.2 - 2.40**

DISCUSSION

As can be seen form the tables, the highest activity was determined for radioactive potassium-40. In the tested samples, its activity ranged from 32 Bq/kg (Table 1) to 123 Bq/kg (Table 2). The values obtained for the concentration of ^{40}K activity are in agreement with the results reported by other authors (Borawska et al., 2013; Pöschl et al., 2011). The ^{40}K activity increased according to the type of honey in the following order: sunflower, flower (polyfloral), linden, meadow, acacia, rapeseed and chestnut. From a biological and ecological point of view, ^{40}K is one of the most important natural radioactive elements. This radionuclide easily moves from the soil to plants and animals, and through foods of plant and animal origin, like honey it can also reach the human body. Since it is in the soil and in the human body, potassium-40 causes external and internal irradiation of all tissues, and especially radiation of soft tissues in the human body. Potassium is under homeostatic control in the body and it is estimated that the annual effective dose received by the human body as the result of the presence of ^{40}K is 165 μSv (Pavlović and Nikezić, 1995). From the obtained results, it can also be concluded that radioactive potassium-40 mostly contributes to the natural radioactivity of honey.

The measured activity of the natural radioisotope in the tested honey samples ranged between < 0.2 and 2.40 Bq/kg. These ^{22}Na activities are common for most of the samples from the nature. It is estimated that the annual level of ^{22}Na intake by ingestion is about 50 Bq which contributes to the annual effective doze of 0.15 μSv (Pavlović and Nikezić, 1995). As a cosmogenic radionuclide that is formed in the constant interaction of cosmic radiation with various atoms and molecules in the atmosphere, earth and water, from the radioecological point of view it is not of great importance for the radiation safety of the biosphere.

The measured activity of the natural radionuclide in honey ranged from 0.82 to 2.0 Bq/kg. The measured values of ^{232}Th activity were in agreement with the values reported by other authors (Dizman et al., 2020). Various accidents at nuclear power plants can lead to a significant increase in the concentration of this radionuclide in food. If ^{232}Th released in this way reaches the food chain, it can be deposited in bone tissue, lungs and liver, and thus increase its radioecological significance in the overall radiation of the population. The average daily intake of ^{232}Th through food in the Republic of Serbia is 1.76 mBq (Đujić, 1995).

When it comes to natural radionuclides in uranium samples, the presence of uranium-238 and radium-226 was registered. The activity of ^{238}U was in the range between < 1 and 31.4 Bq/kg, which is significantly higher than the values

reported by other authors (Đurić and Popović, 2000) while the activity of ^{226}Ra ranged between 1.9 and 15.6 Bq/kg. The presence of these isotopes in honey is of great concern because U-238 and its derivatives Ra-226 and radon-222 pose the greatest risk for human health (Dangić, 1995). The total daily intake of ^{238}U through food is 12.48 mBq and the average daily intake of ^{226}Ra in Serbia is 52.3 mBq which corresponds to the average intake of ^{226}Ra in other countries, and its estimated annual dose is between 20 and 30 μSv (Đujić, 1995). Due to the very long physical and biological half-life, its amount in the body increases over time. The measured U-235 activity was in the range of < 0.2 and 1.61 Bq/kg. In the body, uranium acts as a toxicant because it is a source of ionizing radiation, and also a chemically toxic element. The kidneys are critical organs for natural uranium and its isotopes because if they reach the lungs, they quickly pass into the blood and are eliminated from the body through the kidneys (Mitrović, 2001).

The results of honey contamination measurements, which are shown in Table 1 and Table 2, indicate that the most intense contamination comes from the artificial radionuclide cesium-137. It ranged from < 0.2 to 3.63 Bq/kg. Similar results for ^{137}Cs activity in honey and detection limit for most of the examined samples are reported by Beňová et al. (2019). This activity is particularly important because ^{137}Cs is a biologically important radionuclide which, if it reaches the human body through the food chain, is distributed to all organs almost evenly. The analysis of the ^{137}Cs content in the soil shows that there is a stronger binding of this radionuclide to the surface layers of the soil (0 - 40 cm), while its migration to deeper layers (40 - 80 cm) is significantly slowed down. These results are in full agreement with previous research on the deep penetration of ^{137}Cs, which showed that about 80% of its activity is retained in the surface layer of the soil.

It is interesting to point out the fact related to the radio contamination of bees. Given that honey is a bee product, it would make sense to assume that the bee organism itself will be significantly contaminated. However, since bees have a short lifespan, this contamination cannot in any way affect the bee as a living organism, especially because insects as a species are known to be very resistant to ionizing radiation even at doses of a few tens of Gy, which are otherwise supraleathal for humans (Hadžimuratović et al., 1987). Also, when exposed to microwave radiation, bees did not show any abnormalities related to the ability to fly, orientation, memory and efficiency in performing work (Terzin, 2010).

Many studies of the radiochemical composition of honey (Mihaljev et al., 2001) found multiple links between the mineral composition of honey (con-

tent of natural radionuclides and other micro and macro elements) and its geographical and botanical origin. This means that by applying appropriate statistical methods, certain types of honey can be very successfully identified as well as classified according to their geographical origin. The results indicate that the activity of natural radionuclides in honey varies depending on the geochemical characteristics of the soil, the proximity of industrial plants and that the content of radionuclides depends on the type of vegetation - honey flora (Đurić et al., 1996).

CONCLUSION

The presence of biologically significant radionuclides, both natural (^{238}U, ^{235}U) and artificial (^{137}Cs), was determined in some analyzed honey samples. Therefore, it is necessary to systematically study radioactive substances in bee pastures - their identification, distribution and quantity in the system of soil-honey plant species-honey. Numerous radionuclides found in the nature with the potential to concentrate locally in honey in the levels that are dangerous for the health of the population, call for the need to carry out appropriate systematic research in order to prevent harmful effects of ionizing radiation.

Increasing intake of radionuclides from anthropogenic sources like accidents at nuclear power plants, application of phosphate fertilizers, pollution from thermal power plants and the use of ammunition with "depleted uranium" pose significant risks to public health and environmental protection. Honey bee and its products and specific properties can serve as some kind of "environmental guard" because many changes and pollutants in the nature are quickly repercussed on bees that are very sensitive to any changes in the environment and react immediately to pollution (pesticides, toxic elements, radionuclides) that we normally do not notice. Therefore, honey, as the final product of bees and pollen deserves special attention when considering radioactive contamination.

The obtained results are of great importance for the protection of the environment of the examined areas because these data can be a reference since they represent a "zero state" in the case of future anthropogenic activities that may cause additional contamination. Regarding the determined concentrations of the activity of the tested radionuclides, it can be concluded that honey produced in the Republic of Serbia is a healthy and environmentally safe food and does not pose a radiation risk to human health.

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Author's Contribution:

ŽM and MŽB made contributions to the idea of the publication, organisation of work and writing the manuscript; ŽM and NP did the laboratory analysis; and SJ, MŽB and NP reviewed the manuscript and participated in the final draft of the manuscript.

Competing interest

The authors declare that they have no competing interests.

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THE MOST COMMON CAUSES OF HONEYBEE POISONING

Radomir Ratajac[1*], Jelena Petrović[1], Brankica Kartalović[1], Igor Stojanov[1], Vladimir Polaček[1]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia

Abstract

This paper discusses the most common causes of poisoning of honeybees and other pollinating insects that occur in the Republic of Serbia and the world as well. Some potential ways of pollinator exposure to different poisons and their intake are described. The paper also deals with the methods of testing and assessing toxicity of newly synthesized chemical substances and new formulations, classification of potential toxic substances according to their chemical characteristics and mechanism of action on the insects, symptoms of honeybee poisoning and risk assessment for the uses of pesticides. In the end, the paper looks into the methods of responsible use of pesticides and their toxicity in order to avoid bee poisoning.

Key words: honeybees, toxicity, pesticides

[1*] Corresponding Author: ratajac@niv.ns.ac.rs

NAJČEŠĆI UZROCI TROVANjA MEDONOSNIH PČELA

Radomir Ratajac[1], Jelena Petrović[1], Brankica Kartalović[1],
Igor Stojanov[1], Vladimir Polaček[1]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija

Kratak sadržaj

U ovom radu razmatraju se najčešći uzroci trovanja medonosne pčele i drugih insekata oprašivača koja su evidentna u Republici Srbiji, kao i u svetu. Opisuju se mogući načini izloženosti oprašivača različitim otrovima i putevi unosa istih. Takođe, obrađuje se načini ispitivanja i procene toksičnosti novosintetisanih hemijskih supstanci i novih formulacija pesticida, klasifikacija potencijalnih toksičnih supstanci prema njihovim hemijskim karakteristikama i načinu delovanja na insekte, simptomi trovanja medonosnih pčela i procena rizika upotrebe pesticida. I na kraju, načine odgovorne upotrebe pesticida i mere koje se mogu preduzeti radi smanjenja trovanja pčela agro hemikalijama.

Ključne reči: pčele, toksičnost, pesticidi

INTRODUCTION

Honeybee (*Apis mellifera*) is a cosmopolitan species bred by beekeepers today for the production of commercially valuable products such as honey, wax, propolis, royal jelly, pollen and bee venom. The other reason for beekeeping is pollination, honeybees are the most successful and commercially exploited pollinators in agro-ecosystems. Nowadays, honeybees are bred using organic and conventional method of beekeeping, and in recent years, urban beekeeping has become more widespread in urban areas. The bees are continuously exposed to a wide range of stress factors such as: diseases of various etiologies, parasites, predators, chemical substances from natural and synthetic sources, etc. which are present in the environment (Ostiguy et al., 2019). Foraging worker bees are mostly exposed to adverse environmental influences because they can fly a distance of over 6 km in radius in search for food. In one day, these bees have 12 - 15 excursions and come into contact with pollutants from the environment (Beekman and Ratnieks. 2000). During the search for food, contaminants from the atmosphere remain on the bees' bodies and they are brought to the hive through pollen and nectar, where they reach other

bees in the colony, and the brood. Therefore, worker bees, honey and pollen are often used as bioindicators of environmental pollution by various organic and inorganic compounds (Petrović et al, 2019; Kartalović et al., 2020). The use of a wide range of chemicals in agriculture, alone and / or in combination with other factors, such as elevated temperature, production of hybrid varieties with less pollen and nectar in flowers, has had a devastating effect on honeybees globally. Some of these examples are increased mortality of bee colonies that was recorded in 2006 in the United States (Henry et al., 2012). The mass extinction of bee colonies has been named Colony Collapse Disorder syndrome (CCD) (Retschnig et al., 2015). The collapse of bee colonies is a sudden disappearance of entire bee colonies, with no dead bees in the colony or hive and no kleptoparasites in the extinct hives, regardless of the excess food stored in them. The exact reason for this phenomenon has not yet been clearly explained, but it is believed that several stressors, acting individually and / or synergistically, contribute to the weakening of the health of bees, making them susceptible to the disease. Among systemic insecticides, newer generation pesticides, fipronil and neonicotinoids have been emphasized as the main causes of the collapse of bee colonies since the beginning of their application in the mid-90s of the last century. (Hoshi et al., 2014; Retschnig et al., 2015). This is why the world today is facing a great crisis, which has not only an ecological dimension, but also an economic one, because due to the reduction of the pollinator population, the yields of fruits, vegetables and cereals are decreasing, which directly leads to higher food prices, which affects consumers.

EXPOSURE OF BEES TO PESTICIDES

Different pesticide formulations are used to protect the plant from pests. Depending on the type of pesticide and the recommended method of use, they can be utilized for direct spraying of the plant or for the treatment of soil and seeds. Different methods of application and persistence of applied pesticides play a key role in the exposure of pollinating insects to these chemicals, and these are the main routes of exposure: a direct contact with the chemical during feeding on the treated plant; dust particles contaminated with pesticides, which stick to the bees or are carried to the hive by the wind; contamination of watercourses - surface waters with chemical agents used for field treatment; guttation water; honeydew (secretion); application of pesticides to untreated agricultural crops near the treated field and pesticide residues in pollen by seed treatment (Gaurava et al., 2020).

These different ways of exposing honeybees to pesticides allow contamination of bee colonies, but still the ways bees transmit these chemicals from the

field are various and can be either oral, respiratory or dermal (cuticular).

Oral intake of chemical pesticides from the field is facilitated by foraging worker bees. The plants treated with insecticides produce contaminated nectar and pollen. Some studies have found the presence of high concentrations of various pesticide compounds, including insecticides, fungicides, miticides and herbicides in pollen samples of several crops (Krupke et al., 2012). The bees collect this flower resource carry them to colonies where they continue to use it to feed the bee brood (Wu et al.., 2011). The consequences can be numerous: foraging worker bees die during the collection and transport of contaminated pollen, bees in the hive die during storage and feeding, and broods die by consuming poisonous pollen, which leads to a complete collapse of the colony (Gaurava et al., 2020).

Pesticide formulations, such as dust and fumigants are sprayed by air and can be retained on the body surface of foraging worker bees or absorbed through the trachea in the concentrations sufficient to be toxic to bees (Gaurava et al., 2020). Inhalation of air contaminated with toxic substances causes various abnormalities, such as sudden changes in behavior and degradation of learning abilities (Karise and Mänd, 2015).

Foraging worker bees come into direct contact with pesticides while searching for food, and such chemicals can be lethal even in small quantities. Body wall - the chitinous cuticle of the thorax of honeybees is considered the main route of cuticular exposure to pesticides (OEPP/EPPO, 1992), although in some other studies it has been observed that insect wings are also a potential source of bee exposure (Poquet et al., 2015).

CLASSIFICATION OF TOXIC SUBSTANCES

Toxic chemicals can be classified by the levels of toxicity of substances for bees or based on the origin (pollutants, agrochemicals, medicines).

First, it should be pointed out that there is a great risk of using pesticides due to their acute toxicity to bees, which causes their mortality in a short period of time. Other very significant risks include sublethal effects that can adversely affect hive performance and colony survival in the long run. Pesticide toxicity varies from very high to very low. Substances with very low toxicity are practically non-toxic.

In order to define and categorize toxicity of pesticides during the development of substances, acute oral and contact toxicity tests are performed, according to the methods based on the guidelines issued by the European and Mediterranean Plant Protection Organizations (EPPO), i.e. The Organization for Economic Co-operation and Development (OECD, 1998, 1998a). These

toxicity testing methods are laboratory methods designed to assess the acute oral and contact toxicity of plant protection products and other chemicals on adult worker bees. The method is particularly suitable for phased hazard assessment programs that pesticides pose to bees, based on a hierarchical order, from laboratory toxicity tests to semi-field and field tests. Acute oral and contact toxicity are adverse effects that occur no later than 96 hours after oral or contact administration of a single dose of the test substance. A dose is an amount of the test substance consumed or applied and is expressed as the mass of the test substance per individual (μg/bee). The precise oral dose for each bee cannot be calculated because the bees are fed collectively, instead an average dose can be calculated (fully consumed test substance by the number of individuals - bees). Oral or contact LD50 (mean lethal dose) is a statistically derived single dose of a substance that can cause the death of 50% of animals when administered orally or when it comes into contact with it.

Based on the acute toxicity, the tested substances are classified into the following:

- Substances with very high toxicity to bees, acute toxicity to bees LD50 < 2 μg/bee
- Substances with moderate toxicity to bees, acute toxicity to bees LD50 2 - 10.99 μg/bee
- Substances with low toxicity to bees, acute toxicity to bees LD50 11 - 100 μg/bee.
- Substances that are not toxic to bees, acute toxicity to bees LD50 > 100 μg/bee.

Other methods for testing and assessing the toxicity of chemicals on bees have also been published:

- Guidance document on the honeybee (*Apis mellifera* L.) brood test under semi-field conditions (OECD, 2007). Principle of the test: Shortly before full flowering of the crop and some time before application of the test chemical, small healthy honeybee colonies are initially placed in tunnel tents. The bees are exposed in the tunnel for the period of flowering of the crop (e.g., at least 7 days after application of the product) after which the hives are placed outside the tunnel for the remaining of the study and are free to forage in the field. Over the period of at least 4 weeks after the initial brood assessment, the evaluation of the mortality of honeybees, flight activity, and condition of the colonies and development of the bee brood is done several times. Results are evaluated by comparing the treated colonies with the water treated colonies and with the reference chemical-treated colonies.

- Guidance document on the honeybee (*Apis mellifera* L.) larval toxicity test, single exposure (OECD, 2013). Principle of the test: First instar synchronized larvae (i.e., larvae of the same age) are taken from the comb of three colonies and individually placed into 48 well-plates where a standardized amount of artificial diet is introduced. After three days, a single dose of the test chemical is administered to the larvae with the diet in a range of five increasing concentrations. Mortality was monitored for three next days and the 72 h LD50 is calculated for larvae.
- Guidance Document on honeybee (*Apis mellifera* L.) larval toxicity test, repeated exposure (OECD, 2016). Principle of the test: The preparation of the test and the selection of larvae is the same as for the single exposure. The chemical is administrated to the larvae during three days at a constant concentration equivalent to increasing test chemical doses per larva per day with the diet resulting in a cumulative dose on third day (for each treatment level) in a range of at least five increasing test concentrations, or at one concentration in case of a limit test. Mortality and other observations/ abnormal effects are recorded daily from during administration and on ninth and 16th day after administration. The NOEC (No Observed Effect Concentration) /NOED (No Observed Effect Dose) and, if data allows, the EC50/ED50, and/or any ECx/EDx are determined).
- Guidance Document on honeybee (*Apis mellifera* L.) chronic oral toxicity test (10-day feeding) (OECD, 2017). Principle of the test: Aqueous sucrose solution containing the test chemical is used to expose young bees, during a period of 10 days (*ad libitum*). Behavioral abnormalities and mortality are observed and recorded daily. The chronic effects of the test chemical are evaluated by comparing the results of the test chemical treated group to those of the respective control group. Following endpoints are determined by using this test: • LC50 (median Lethal Concentration) and the LDD50 (median Lethal Dietary Dose) values after 10 days of exposure. • NOEC and NOEDD (No Observed Effect Dietary Dose). In some cases, a limit test may be performed (e.g., when a test chemical is expected to be of low toxicity or when a test chemical is poorly soluble), in order to demonstrate that the NOEDD is greater than or equal to the limit dose tested, and the LDD50 is greater than the limit dose tested, if no effects are observed in the study.
- Guidance Document on honeybee (*Apis mellifera* L.) homing flight test, using single oral exposure to sublethal doses of test chemical (OECD, 2021). Principle of the test: This test method measures the effect of single sublethal oral doses of a test chemical (under controlled conditions) on the

homing success of forager honeybees (under simulated field realistic conditions). Foragers are released 1 km away from the colony, and the homing success of chemically exposed versus non-exposed foragers is compared. This is achieved by monitoring the experimental bees with radio-frequency identification (RFID) tagging technology. The test is done in three replicates. The objective is to determine a NOED on homing success from all the doses of the chemical tested.

The second type of classification is based on the type of toxins that come from different sources.

Environmental pollutants

According to their origin, they are divided into natural and artificial-anthropogenic.

Carbon dioxide

The so-called global warming is changing meteorological factors and even the climate with unforeseeable consequences for the entire living world on the globe. The impact of pollutants on the survival of bees and other insects is also evident, with carbon dioxide potentially being toxic. The studies of the impact of carbon dioxide on honeybees have shown that CO2 can have some toxic effects on bee health at the individual and colony level. Changes were observed in the lifespan of bees (shorter), the amount of pollen collected during the flowering season (reduced), the narcotic effect in terms of reduced activity at elevated CO2 concentrations (Maini et al., 2010) and earlier oviposition of the queen bee (Gaurava et al., 2020).

Heavy metals

Due to the growing anthropogenic impact, heavy metals are one of the main pollutants in the environment. Unlike organic pollutants, heavy metals are not subject to degradation and they accumulate not only in the environment, but also in the living systems. The most common toxic elements known for their high levels of toxicity to living organisms, and its wide distribution, are cadmium, lead, mercury, arsenic, chromium, nickel, as well as micro elements such as copper, iron, zinc, manganese, cobalt and selenium. However, they are necessary for various biochemical and physiological processes in low concentrations, but when their concentrations are higher, they may become harmful.

Heavy metals are among the most important potentially harmful pollutants and their presence can affect various physiological and metabolic processes. Toxicity of heavy metals is manifested by interaction with biomolecules in several ways: changes in the conformation of biomolecules, blocking of essential functional groups of biomolecules, replacement of essential metal ions in biomolecules, oxidative damage of biomolecules.

Various studies have shown that high concentrations of cadmium, copper and lead have a toxic effect on bees, which can lead to oxidative stress of individuals and disrupt the homeostasis of micro elements and detoxification of toxic metals, which then results in changes in the behavior and diet of bees. Cadmium and copper are actively absorbed by the root system of the plant from the soil and can be found in nectar and pollen, and bees usually come into contact with lead through contaminated surfaces where lead settles (Nikolić et al., 2016).

Medicinal - active substances used in beekeeping

Proper and timely use of veterinary preparations medications will not only protect bee colonies from diseases and pests, but will also prevent contamination of honey, wax, propolis and other bee products.

In the Republic of Serbia, the use of antibiotics in beekeeping is strictly forbidden because the consequences can be severe: destruction of saprophytic microflora and violation of bee colony immunity, concealment of diseases, frequent relapses, resistance to frequent administration of antibiotics, antibiotic residues and secondary metabolites in bee products (Kartalović et al., 2020).

To control the most widespread disease of honeybees caused by ectoparasite *Varroa destructor* in the Republic of Serbia, registered preparations based on: coumaphos (organophosphorus compound), fluvalinate (tau-fluvalinate - pyrethroid), formic acid, as well as active substances of essential oils, camphor oil and the oil itself, menthol, eucalyptus oil (ALIMS, 2021). In addition, other organic acids such as lactic and oxalic acid are used. These chemicals have proved to be far more successful than other treatments, but at the same time their toxicity to bees has sometimes been neglected or studied less. However, the emergence of resistant mite populations has resulted in a sharp increase in the use of formic acid and oxalic acid in practice, as they are natural varocides that are also normally found in honey (Bogdanov, 2006). Organic acids are quite effective in controlling *Varroa* mites, but there haven't been many studies to determine their negative impact on honeybees. Schneider et al. (2012), they pointed out the harmful effects of organic acids on honeybees, which include

the following: negative impact on brood development, lower physical fitness of the treated colony, increased mortality, reduced division of labor and reduced cleaning of hives and increased self-cleaning.

Toxicity of formic acid

Formic acid inhibits energy metabolism, i.e., the formation of hypoxic metabolism and histotoxic hypoxia (Keyhani and Keyhani, 1980). Also, formic acid can have an excitatory effect on the neurons of the parasite and penetrate through the thin exoskeleton of the parasite and thus cause their additional irritation and death. The selectivity of action is based on the difference in the thickness of the cuticula of the bee and a tick that parasitizes on it. Bees can tolerate 250 times the dose of formic acid compared to *Varroa* (ALIMS 2021a). When used in the recommended doses, i.e., according to the instructions for the drug, it is not harmful to bees. Data can be found in the literature that bees may experience increased buzzing and agitation, which usually disappears quickly. However, formic acid can cause a variety of toxicity symptoms in honeybees, including reduced worker bee life and lower brood survival rates (Underwood and Currie, 2003). It can have a toxic effect on the larvae in a covered brood, which then perish, and it can also have a detrimental effect on the open brood and hatching of bees. There is a possibility that (due to toxic effects) a certain number of queen bees might get lost. Other negative effects of formic acid treatment on bee colonies mainly include increased number of dead bees in front of the colony during the treatment period, rejection of queen bee, reduction in drone eggs. In addition, worker bees may be rejected from the colony and honey yield from treated colonies can be relatively lower (Gaurava et al., 2020).

Oxalic acid toxicity

The mechanism by which oxalic acid acts on *Varroa* has not yet been fully explained. It has been observed that treatment with this acid (sugar solution of oxalic acid) leads to an increase in apoptosis of middle intestine cells of bees, so it cannot feed and therefore dies of hunger (Gregorc and Škerl, 2007). Multiple use of oxalic acid, most commonly through sugar syrup, can lead to increased queen bee mortality and a reduction in the number of sealed broods (Higes et al., 1999). In the treated colonies, it has been observed that, in the early stages of life, worker bees show abnormal behavior that depends on the age of the individual. The behavior of bees, which depends on their age, is chronologically normal, but the intensity is different, and some stages appear

earlier than normal. Treated bees show increased self-care, decreased activity, especially care bees (Gaurava et al., 2020).

Agrochemicals – Pesticides

Pesticides are chemical agents used to control plant pathogens, against harmful insects, nematodes, rodents and birds, algae and to control weeds or regulate plant growth, destroy fungi, and kill insects and other organisms that transmit infectious diseases in humans and animals.

Insecticide toxicity

Insecticides have been used since the early 1940's to effectively control pests and are usually divided according to their chemical composition and purpose. Insecticides from the group of organochlorine and organophosphorus insecticides, are significantly less used today or their use is prohibited (Petrović et al, 2021). Honeybees are susceptible to many insecticides, and various harmful effects of these insecticides are believed to be the main reason for the decline in the global bee population, the CCD (Retschnig et al., 2015).

Organochlorine insecticides

Organochlorine compounds are chemically derived from chlorine derivatives of aliphatic and aromatic hydrocarbons (DDT, Eldrin, Dieldrin, Lindane, etc.). The characteristic of these compounds is that they are very lipophilic, and they accumulate in tissues rich in lipids, which leads to bioaccumulation and an increase in the concentration of pesticides through food chain – biomagnification (Petrović et al., 2021). Biological activity of organochlorine compounds is aimed at stimulating the nervous system, resulting in disorders in the transmission of nerve impulses. Due to their ability to accumulate and remain in the environment for a long time (POPs - persistent organic pollutants), most organochlorine insecticides are banned for use and withdrawn from the market.

Organophosphate (OP) and carbamate insecticides

Two widely used groups of insecticides are organophosphates (esters of phosphoric acid: methyl parathion, phorate, coumaphos, etc) and carbamates (organic compounds derived from carbamic acid: carbaryl, carbofuran, aldicarb, etc). Both groups of insecticides affect insects in a similar way as acetyl

cholinesterase (AChE) inhibitors (OP irreversible, carbamates reversible), resulting in severe hyperexcitation and convulsions, leading to paralysis and death (Dulin et al., 2012). The values of LD50 topical toxicity for the active substances of these two classes of insecticides are in a wide range (0.094 (oxamyl) to 20 (coumaphos) μg/bee) (EPA, 2021). Toxic symptoms of organophosphate are irregular and disoriented movement of bees, joined wings, enlarged abdomen, tongue sticking out with regurgitation of food, and death. Toxic symptoms of carbamate include improper movement of bees, with swelling (numbness) and paralysis, interruption in the brood cycle, while queen bee stops laying eggs, and eventually most bees die in colonies (Gaurava et al., 2020).

Pyrethroids

Pyrethrin insecticides, produced from the flowers of pyrethrum (*Chrisanthemum inerariaefolium*) are a widely used group of insecticidal compounds. Although pyrethrin is of natural origin, it is known that these chemicals are very toxic to bees (LD_{50} = 0.022 - 0.21 μg/bee) (EPA, 2021). In addition to pyrethrin and pyrethroids insecticides show their effect on parasites by altering the permeability of voltage sodium channels in nerve cell membranes, leading to membrane depolarization, i.e., hyperexcitability, and as a result, a rapid paralysis of individuals ("knock down effect") and death. In addition, they can act on the postsynaptic membrane and on nicotine, GABA (gamma-aminobutyric acid) and glutamine receptors, as well as on voltage-gated calcium channels. Bees have greater tolerance to some pyrethroids due to their rapid detoxification by the cytochrome P450 enzyme. Tau-fluvalinate, a widespread miticide, is less toxic and it is safer for bees, but in higher concentrations, this chemical has a negative effect on the health of different classes of bee colonies, as it causes temporary disturbance and weight loss in queen bees (Haarmann et al., 2002). It has also been observed that drones are exposed to tau-fluvalinate during development with less chance of reaching sexual maturity (Rinderer et al., 1999). Toxic symptoms of synthetic pyrethroids are improper movement of bees and paralysis, regurgitation of food intake, and eventually many bees die between the area where they look for food and the colony (Gaurava et al., 2020).

Neonicotinoids

In the past, tobacco extract was used for protection against pests, but due to its toxicity, it is now not used almost at all. In the studies that dealt with the toxicity of nicotine, it was found that there is 23 ppm of nicotine in the pollen

of the tobacco plant (*Nicotiana tabacum*) and 0.1 - 5 ppm in nectar. It was also found that adult bees successfully detoxify nicotine in nectar, while larvae are sensitive to nicotine and usually die in the third or fourth stage of development at the concentration of 50 ppm (Singaravelan et al., 2006). Nowadays, there are more modern protective substances, some of which are chemically similar to nicotine, but have greater efficiency in the fight against harmful insects, and relatively lower toxicity to the human body - neonicotinoids: nitroguanidine neonicotinoids; nitromethylene neonicotinoids and pyridyl methylamine neonicotinoids. There are many advantages to neonicotinoids compared to pyrethroid, organophosphate and carbamate insecticides and people are increasingly replacing them worldwide. Neonicotinoids act similarly to natural products - nicotine, acetylcholine, epibatidine, as agonists of postsynaptic nicotinic acetylcholine receptors (nAChR) of insects. Neonicotinoids are one hundred times more selective for nAChR insects than vertebrates (Tomizawa and Casida, 2005). Some toxicity studies of these compounds have indicated that these insecticides are potentially very dangerous and may be one of the causes of CCD (Hoshi et al., 2014). Nitroguanidine neonicotinoids have been reported to be highly toxic to bees, with toxicity levels ranging from 0.0038 (imidacloprid) to 0.024 (thiamethoxam) μg/bee (EPA, 2021). Insecticides from the nitroguanidine group also show their toxic effect by reducing the ability of queen bees to return to the hive. The cyano-substituted neonicotinoids exhibited a much lower toxicity with LD50 values for acetamiprid and thiacloprid of 7.1 and 17.94 μg/bee, respectively (EPA, 2021). This relatively low toxicity is probably a result of detoxification under the impact of the cytochrome P450 enzyme (Blacquiére et al., 2012).

In 2013, the European Commission (EU Regulation No. 485/2013) and Serbia temporarily introduced a restriction on the use of clothianidin, thiamethoxam and imidacloprid in order to reduce their impact on bees. The use and sale of seeds treated with plant protection products containing these active substances, except for seeds used in greenhouses, is prohibited.

Phenylpyrazoles

The main representative of this group of insecticides is fipronil, a broad-spectrum insecticide. It was manufactured in 1987 and originally developed for the use in pest control in agriculture and for public health (Zhang et al., 2016). It acts by inhibiting the GABA complex (main inhibitory neurotransmitter) and by binding to chlorine channels thus blocking pre and postsynaptic transfer of chloride ions across the cell membrane. In this way, it in-

hibits the transmission of nerve impulses between nerve cells, which leads to uncontrolled activity of the central nervous system and insect death by hyperexcitation (Islam and Lynch, 2012). Fipronil has a strong affinity towards invertebrate GABAergic receptors, which makes it more toxic to insects than to mammals (Narahashi et al., 2007). Fipronil is very toxic to bees (LD50 = 0.0218 μg/larvae, 0.004 μg/bee) (EPA, 2021), causing restlessness, tremor, and paralysis. Bees that were exposed to lethal or sublethal doses showed reduced motor activity. Exposure of bee colonies to sublethal concentrations of fipronil led to a reduced number of hatched eggs, a smaller number of worker bee eggs and fewer larvae and pupae, while adult bees were lethargic, colonies were becoming slowly weaker, and bees were leaving the hive (Zaluski et al., 2015).

The European Commission (EU Regulation No. 781/2013a) and Serbia introduced a restriction on the use of fipronil due to the high risk for bees in 2013. The use and sale of seeds treated with plant protection products containing fipronil is prohibited, except for the seeds used in greenhouses and for some types of onions and cabbage that are grown in fields and harvested before flowering.

Fungicide toxicity

It is generally accepted that fungicides are not toxic to bees, so they are sometimes applied during the flowering of a plant that coincides with the maximum activity of bees, as residues are often found in the pollen of honeybees (Kubik et al., 1999). But beekeepers have reported brood losses in the larval and pupae stages that coincide with the use of fungicides during flowering. Malformations also occur, there are young bees without wings, which gather at the bottom and at the entrance of the hive. It has also been found that the application of fungicides causes hypothermia in adult honeybees (Gaurava et al., 2020). Toxicity levels for various fungicides range from LD50 10 to as much as > 200 μg/bee (EPA, 2021).

Herbicide toxicity

The level of herbicide toxicity is known to be very low for most insects and therefore these pesticides are applied without any insect restrictions. High concentrations of herbicides can have toxic effects on honeybees, LD_{50} values vary in the range 15 – > 100 μg/bee (EPA, 2021). For a widely used herbicide, paraquat has been reported to be toxic to bees in the laboratory, causing a tenfold shorter life span of a worker bee than normal when 15 μg of active

substance per bee is applied. When treating crops at a concentration of 4.5 kg of active substance/ha, bee death occurs within 3 days. On the other hand, the use of herbicides can reduce the number of plant species thus reducing grazing - flower resources (Gaurava et al., 2020).

SYMPTOMS OF BEE POISONING

Symptoms of bees poisoning will depend on the toxicity of the substance, duration, and place of exposure of bees and colonies:

- If bees are exposed directly in the field to pesticides of high toxicity, during food collection, many dead bees and other insects will be found in the fields, whereas only a part of them will manage to return to their hives. Hives themselves might be located nearby the fields treated with highly toxic chemicals for bees, which sometimes causes massive death of entire colonies. Usually, stronger colonies will be affected more than weaker ones, as they are more active in collecting food.
- The presence of a large number of dead and dying bees in front of the hive, and at the entrance to the hive is one of the obvious signs that the foraging bees were in contact with the toxic substance. Also, sticky, thick, dark liquid may be present at the entrance due to regurgitation of collected nectar in poisoned bees.
- Bees can change their behavior due to contact with contaminated food and be very upset, so they can be very aggressive when examined by beekeepers.
- In addition, the following prolong symptoms may occur, with less toxic substances and / or less exposure to toxic substances: twitching movements, dizziness, slow activity, crawling and paralysis of bees, loss of ability to fly and usually death occurs in only a few days (2 - 3 days).
- When inspecting the hive, the following signs may indicate bee intoxication: poor egg-laying patterns or abnormal queen bee behavior, disruption of the brood cycle (the stage of young bees) or the variegated of the brood (Gaurava et al., 2020).

RISK ASSESSMENT OF PESTICIDE USE

Risk assessment of pesticide effects on the bee colony is complex and cannot be based solely on data on oral and contact acute toxicity expressed through the LD50, which is a classic toxicological test to investigate the effects of pesticides on bees (OEPP/EPPO, 1992). This approach to testing the effects of pesticides on bees is necessary, but on the other hand, it is not sufficient for

testing pesticides that have a different route of exposure into the bee, i.e., bee colony, a different mechanism of action and some of them could cause harmful chronic effects on bees. Therefore, more information on the toxicity of pesticides to adult bees and their developmental forms (testing and sublethal doses, etc.), provides a better insight into the real risk potential of their use for bee colonies. Therefore, Colin et al. (2004) believe that exposure of the entire bee colony to pesticides, through a chronic toxicity test could quantify the effect of pesticides on bees way better, and above all systemic insecticides. In order to better understand the impact of pesticides on bees and the bee colonies, testing the effect of pesticides on bee larvae grown *in vitro* is also being conducted (Aupinel et al., 2005).

The following indicators should be taken into consideration for bees' risk assessment (Mirjanić and Mitrić, 2012):

- For adults: LD15, LD50; LC15, LC50; LDD50; NOED, NOEC and NOEDD
- For larvae: LD50; LC50; NOEL - No Observable Effect Level and LOEC- Lowest Observed Effect Concentration
- Pesticide application rate: PEC- Predicted Environmental Concentration; PNEC- Predicted No Effect Concentration; TER - Toxicity Exposure Ratio (TER = LD50/PEC)
- HQ1- hazard quotient (HQ1=application rate/LD50); HQ2 for adults (PEC/NOEC); HQ3 for larvae (PEC /NOEC)

Despite all these data collected during the research of a newly synthesized substance or new formulation, risk assessment in field conditions is sometimes impossible to determine, because the impacts of various factors, which are important for the exposure of bees as individuals or as colonies increase tenfold, starting with the assessment of the amount of pesticides used per unit area, manner and time of use, atmospheric conditions, the condition of bees and colonies, etc... Sometimes, some important facts are missing. This all implies that the research in the field of assessment of the impact of pesticides on bees must continue in every respect, especially in development of software programs that can encompass all databases on pesticide toxicity and their interaction and bee biology and provide the most objective insight into toxicodynamic and toxicokinetic model for bees.

MEASURES THAT CAN BE TAKEN IN ORDER TO REDUCE BEE POISONING BY AGROCHEMICALS

Measures that can be taken in order to reduce bee poisoning by agrochemicals are the following:

- Pesticides that are registered on our market should be used exclusively according to the manufacturer's instructions, in the prescribed amount in quiet weather and prevent "drift" of pesticides, i.e., application to surrounding area, do not apply above 25 °C, and use them only when there is a need for them.
- Do not apply pesticides when the crop is in the flowering stage. The use of pesticides (toxic to bees) during flowering is prohibited according to the Law of Plant Protection Products (Official Gazette of RS, 17/2019). Avoid spraying weeds in during the flowering phase or remove weed flowers before the treatment.
- Prevent surface water pollution by rinsing sprinklers and disposing of used packaging of protective agents in canals and watercourses, etc., because in this way the water used by bees in the summer to cool the colony in the hive is contaminated.
- Farmers who spray plants need to inform the beekeepers or beekeeping associations in the surrounding area about it at least two days before the treatment. All beehives located less than 5 km away from the treated area are endangered. The informed beekeepers must move their hives or close them. A beekeeper is obliged to display a board with his address and telephone number next to the bee yard. Due to the frequent poisoning of bees in cultivated orchards, in order to avoid misunderstandings, damage and death of bee colonies that can occur during pollination, so it is necessary to define the relations between fruit growers and beekeepers in advance.
- In order to protect the bees, and avoid direct contact, it is best to spray in the early evening (most pollinators are active from 8 am to 5 pm), which would allow easily degradable substances to partially decompose during the night, or 2 hours after the sunset or up to two hours before sunrise.
- It is important to make the right choice of pesticides in terms of less toxicity for bees, optimal dosage, and optimal choice of formulation, avoid the use of microencapsulated insecticide formulations, as well as powder forms, ultra-small volume formulations because they are much more available to individuals and bee colonies. The use of liquid formulations, emulsions, granular pesticide formulations is much less dangerous, because the potential for bee exposure is reduced (Gaurava et al., 2020).

CONCLUSION

All the above mentioned indicates that bees have great ecological, and economic importance, and that is why their conservation is crucial and of great significance. In the last few decades, bees have been exposed to increased levels of pollution, which alongside poor nutrition and pathogens contribute to the weakening of bee populations in Europe and the world. Since there is no assessment of the impact of a large number of stressors on bees, it is recommended to work on identification of these factors and their interaction, as well as the assessment of lethal / sublethal effects of various pollutants on bees. The stress to which bees are exposed is the impact of the environment which disturbs or weakens the structure and functioning of the organisms and endangers their survival. The stress response can be studied at different levels of organization: molecular, cellular, histological, physiological, environmental, and social. Studying the mechanisms of adaptation to stressful and extreme environmental conditions provides a basis for solving health problems, enables toxicological risk assessment and the use of bioindications to monitor global changes in the environment.

The pesticides used in agriculture, environmental pollutants, as well as medicines and other substances used in health care and hygiene of bee colonies pose a constant risk in beekeeping, not only because they can endanger the honeybee and the bee colony, but also because that they can contaminate bee products. Proper application of registered pesticides and drugs and compliance with the norms of good agronomic and veterinary practice reduces the possibility of pesticides coming into contact with the bees, i.e., side effects. Systemic insecticides are particularly dangerous and therefore it is necessary to work on the constant development of pesticide risk assessment for bees and follow modern legislation in the field of pesticide toxicology.

Furthermore, farmers and beekeepers need to be continuously educated in order to raise awareness about the ecological and economic importance of bees, harmfulness of various chemicals used in agriculture and beekeeping, with the aim to preserve and develop bee communities, i.e. the biocenosis.

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Author's Contribution:

RR, JP and VP made contributions to conception of the article, involved in data collection and drafting the manuscript. RR, JP and BK contributed with data about residue in bee products (organic and inorganic compounds) and toxicity of chemicals use in agriculture to bees, IS and VP did data processing about diseases of bees various etiologies. Revised the manuscript critically and together with RR, JP, BK, IS and VP prepared the final draft of the manuscript. All authors read and approved the final manuscript

Competing interest

The authors declare that they have no competing interests.

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MAIN RISK FACTORS OF AMERICAN FOULBROOD SPREADING IN HONEY BEES IN SERBIA

Vladimir Polaček[1*], Biljana Đurđević[1], Jasna Prodanov-Radulović[1], Jelena Petrović[1], Radomir Ratajac[1], Sandra Jakšić[1], Milica Živkov-Baloš[1]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia

Abstract

American foulbrood (AFB) is one of the most important contagious honey bee diseases. In Serbia, it is mandatory to report AFB, and this disease is registered in our country every year. Starting from 2018, active surveillance of the presence of the AFB has been conducted throughout the country. The paper analyses the data on the occurrence of AFB during the period between 2019 and 2021 from official disease reports in the National Animal Disease Notification System "VetUp". Results of this research indicate that AFB appears every year in the Republic of Serbia, despite the measures that are being applied. The results indicate that in 36 settlements in the country, this disease has reoccurred in the same localities in the observed period. Namely, in 17 localities AFB occurred consecutively in the 2019 - 2020 period , while in 21 localities the disease was re-registered consecutively in the period between 2020 and 2021 (until November 6th, 2021), and it reoccurred in 2021 in 9 localities, compared to the registered cases of AFB in 2019. It was found that the disease has consecutively been reoccurring in 5 location between 2019 and 2021 in the same locations. The fact that the disease has been occurring for several years in the same places speaks in favour of the fact that the control measures applied in the control of this disease are not effective enough. Continuing education of beekeepers, veterinarians and veterinary inspectors in the field of diagnosis and effective decontamination and neutralization of all potential sources of AFB reinfections, revision of current legislation, as well as raising awareness of the importance of early diagnosis of as many cases of this disease as possible are the key factors in successful AFB control.

Key words: American foulbrood, *Paenibacillus larvae*, honey bees, bee diseases, Serbia

[1*] Corresponding Author: vlade@niv.ns.ac.rs

GLAVNI RIZICI ŠIRENJA AMERIČKE KUGE PČELINJEG LEGLA U REPUBLICI SRBIJI

Vladimir Polaček[1*], Biljana Đurđević[1], Jasna Prodanov-Radulović[1], Jelena Petrović[1], Radomir Ratajac[1], Sandra Jakšić[1], Milica Živkov-Baloš[1]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija

Kratak sadržaj

Američka kuga pčelinjeg legla (AKPL) je jedna od najznačajnijih infektivnih bolesti medonosne pčele. Ova bolest je obavezna za prijavljivanje i svake godine se javlja u Srbiji. Počev od 2018. godine, na teritoriji čitave zemlje počinje da se primenjuje aktivni nadzor na prisustvo AKPL. Ovaj rad analizira zvanične podatke Uprave za veterinu - sistema „Vet-up" o pojavi AKPL u periodu od 2019 do 2021. Rezultati ovog istraživanja ukazuju na to da se AKPL pojavljuje u Srbiji svake godine, bez obzira na mere koje se primenjuju u kontroli ove bolesti. Takođe, rezultati pokazuju da se u 36 naselja AKPL ponovno javlja, u posmatranom periodu. Na 17 lokaliteta se ponovno javlja u periodu 2019 -2020., dok se na 21 lokacije AKP je ponovno registrovana u periodu 2020 - 2021. (posmatrano do 6.11.2021.). Na 9 lokaliteta bolest je ponovno registrovana 2021. godine u odnosu na registrovane slučaje AKPL u 2019. godini, dok se na 5 lokaliteta u Srbiji AKPL pojavljuje svake godine, počev od 2019. do 2021. godine. Činjenica da se bolest pojavljuje više godina na istim lokalitetima govori u prilog da mere koje se primenjuju u kontroli ove bolesti nisu dovoljno efikasne. Neprekidna edukacija pčelara, veterinara i veterinarskih inspektora na polju dijagnostike ove bolesti, efikasne dekontaminacije i neutralizacije svih potencijalnih izvora infekcije AKPL, revizija važeće legislative koja reguliše ovu oblast, kao i podizanje svesti o značaju rane dijagnostike što većeg broja slučajeva bolesti su ključni faktori u uspešnoj kontroli ove bolesti.

Ključne reči: Američka kuga pčelinjeg legla, *Paenibacilus larvae*, medonosna pčela, bolesti pčela, Srbija

INTRODUCTION

American foulbrood is one of the most severe infectious diseases of the honey bees (Beims et al., 2020; Djukic et al., 2014; Genersch, 2010). It is caused

by the spore-forming, Gram-positive rod-shaped bacterium *Paenibacillus larvae* (De Graaf et al., 2013; Forsgren et al., 2018). The spores are the only transmissive stage of the bacteria. They are highly resistant and can remain infectious for more than 35 years (Dobbelaere et al., 2001). Long-lived endospores are an infectious form and only bee larvae younger than 36 h are susceptible. Oral uptake of about ten spores is sufficient to initiate a fatal intestinal infection in bee larvae (Genersch, 2010). After germination, *P. larvae* massively proliferate the larval midgut. The vegetative cells breach the epithelium and invade the haemocoel of bee larvae. This invasion coincides with the death of infected larvae, which are subsequently decomposed into a brown glue-like liquid. The emerging ropy mass dries and develops into a highly contagious scale, which contains a vast number of *P. larvae* spores (Beims et al., 2020; Forsgren et al., 2018; Genersch, 2010). Burning colonies and contaminated hive material are widely considered to be the only workable control measure for diseased colonies. Thus, AFB is a serious problem in apiculture and causes considerable economic loss to beekeepers all over the world (Genersch, 2008; Genersch, 2010). Nowadays, the existence of five different genotypes (ERIC I-V) of *P. larvae* has been determined (Beims et al., 2020; Žugelj et al., 2021). Genotypes of *P. larvae* are differed by the level of virulence. ERIC I is a slow killer, ERIC III a medium fast killer, while ERIC II and IV represent fast killers. While larvae infected with genotypes ERIC II to ERIC IV were killed within only 6 to 7 days, it took *P. larvae* ERIC I around 12 to 14 days to kill all the infected individuals. It has been proposed that the fast-killing phenotype allows nurse bees to remove infected larvae more efficiently. Larvae infected with slow killing *P. larvae* die in cells which are already capped. This apparently reduces the effect of hygienic cleaning by nurse bees. As a result, the infected larvae remain in the cell, convert into infectious spores and thus contribute to disease progression within and beyond the colony (Rauch et al., 2009). EPIC V genotype has recently been discovered in honey samples originating from Spain (Beims et al., 2020).

According Statistical Office of Republic of Serbia, 980,000 beehives were registered in whole country in 2020 (Anon, 2021), but according Association of Beekeeping Organizations of Serbia (SPOS) the number of registered beehives in 2021 is more than 1,500,000 and therefore is a significant branch of agriculture. Reporting AFB in Serbia is mandatory. Since 2005, AFB has been the second most frequently reported disease, according to National Animal Diseases Notification System –"Vetup", with 1,252 total number (average annual number 86) of registered AFB cases in the 2005 to 2018 period, (Polaček et al., 2019.) Starting from 2018, active monitoring of all beehives has been car-

ried out in the Republic of Serbia in the diameter of 3 km from the bee yards where AFB was confirmed by laboratory diagnostics in the previous year. The plan involves clinical examinations of all beehives within a three-kilometre diameter, sampling of enclosed honey combs from bee colonies susceptible to AFB and sending samples to laboratory analysis (Anon, 2021). The rulebook regulating this issue has not changed since 1988 (Anon, 1988). The measures applied in bee yards where this disease is found include destruction of the diseased colony, disinfection, quarantine and compensation of the market value of the bee colony and hive. The treatment of diseased colonies or the application of shook swarm method are not allowed. Although active monitoring of the presence of ABF in Serbia is being carried out, there is an impression that, apart from registering cases, the number of cases of this disease has not decreased in recent years. On the contrary, it is increasing. The aim of this paper is to analyse the data on the occurrence of AFB in Serbia, and analyse the most important factors in the spread of this disease in the Republic of Serbia.

MATERIAL AND METHODS

The paper analyses the data on the occurrence of AFB during the 2019 - 2021 period from the official disease reports in the National Animal Disease Notification System "VetUp". The data were analysed using ESRI ArcGis Map 10.8, ESRI ArcGIS Online, ESRI ArcGIS Pro 2.8, Microsoft Excel, and Microsoft Access 365.

RESULTS

The results show the occurrence of ASF between 2019 and 2021. This disease was recorded each year during the observed period. During 2019, 86 cases of ASF were found in 69 locations in the Republic of Serbia, while there were 113 cases of ASF in 83 locations during 2020. Finally, by November 6th, in 2021, there were 107 cases of ASF at 69 locations. Figure 1 shows the spatial distribution of the cases for the observed period. Namely, AFB appeared consecutively in the 2019 - 2020 period in 17 localities and in 21 localities the disease was re-registered consecutively in the period between 2020 - 2021 (until November 6th, 2021), while in 9 localities this disease reappeared in 2021, compared to the registered AFB cases in 2019. Table 2 shows that at 5 localities in the Republic of Serbia (Bukovik, Trnava, Šabac, Azbresnica, Novi Pazar) it occurred consecutively every year in the 2019 - 2021 period.

Table 1. Number of registered AFB cases in Serbia from 2019 to 2021 (until November 6th, 2021)

Year	Number of places with AFB outbreaks	Number of AFB registered cases
2019	69	86
2020	83	113
2021	69	107

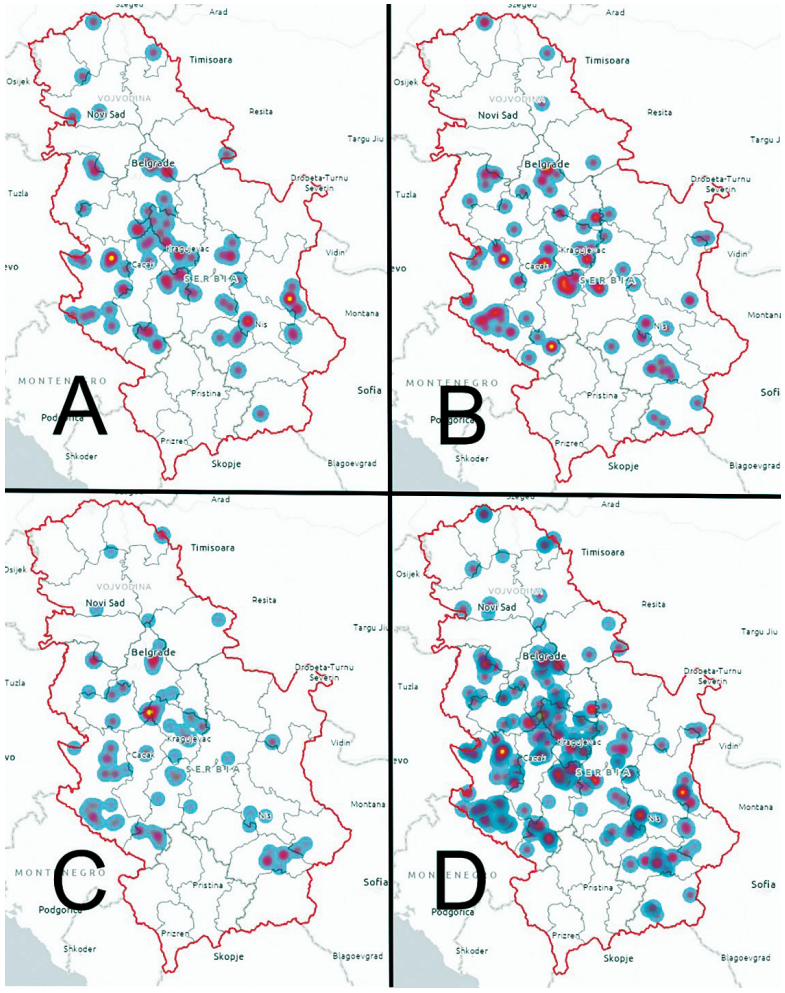

Figure 1. A - AFB outbreaks in 2019; B - AFB outbreaks in 2020; C - AFB outbreaks in 2021; D - Total AFB outbreaks from 2019 to 2021 (until November 6th, 2021)

Table 2. The number of repeated places with AFB outbreaks in at last two years in Serbia from 2019 to 2021 (until November 6th, 2021)

Municipality	Place	Repeated AFB outbreaks 2019-2020	Repeated AFB out-breaks 2020-2021	Repeated AFB out-breaks 2019-2021
Aranđelovac	Bukovik	■	■	■
Aranđelovac	Garašani			■
Bajina Bašta	Perućac			■
Beograd Čukarica	Beograd Čukarica	■		
Beograd-Grocka	Zaklopača		■	
Beograd-Rakovica	Beograd Rakovica		■	
Beograd-Voždovac	Beograd Voždovac		■	
Čačak	Čačak		■	
Čačak	Preljina	■		
Čačak	Trnava	■	■	■
Čajetina	Drenova			■
Leskovac	Strojkovce		■	
Kikinda	Kikinda	■		
Knjaževac	Knjaževac	■		
Kragujevac-city	Donja Sabatna	■		
Kragujevac-city	Kragujevac		■	
Kragujevac-city	Poskurice		■	
Kraljevo	Dragosinjci		■	
Kraljevo	Kraljevo		■	
Kraljevo	Jarčujak	■		
Kraljevo	Žiča	■		
Merošina	Azbresnica	■	■	■
Merošina	Dudolajce	■		
Nova Varoš	Bukovik	■		■

Municipality	Place	Repeated AFB outbreaks 2019-2020	Repeated AFB outbreaks 2020-2021	Repeated AFB outbreaks 2019-2021
Nova Varoš	Vilovi	■		
Novi Pazar	Novi Pazar	■	■	■
Paračin	Paraćin		■	
Prijepolje	Divci		■	
Prijepolje	Kosatica		■	
Rača	Rača	■		
Rača	Viševac		■	
Šabac	Šabac	■	■	■
Subotica	Subotica	■		
Sjenica	Sjenica		■	
Ub	Pambukovica		■	
Užice	Trnava		■	
Total number of repeated places with AFB outbreaks		**17**	**21**	**9**

DISCUSSION

Results of this research indicate that AFB reoccurs in the Republic of Serbia every year, despite the measures that are being applied. The fact that the disease has been occurring in the same places for several years speaks in favour of the fact that the control measures applied in the control of this disease are not effective enough.

It has been pointed out that the endospores of the pathogen itself are very resistant in the external environment. The choice of disinfectants for disinfecting the apiaries and accessories is therefore very important. The data about effective disinfectant against *P. larvae* endospores in literature are scarce. Dobbelaere et al. (2001) examined the efficiency of various disinfectants on the efficiency of wood disinfection, and the material commonly used for building hives. They found that disinfectants based on sodium hypochlorite and the products based on the combination of glutaraldehyde and formaldehyde destroy 100% of AFB spores for 30 min only if used in concentrations above 50% in working solutions. The use of disinfected compounds based on amphoteric

compounds (quateral ammonium compounds) did not result in a decrease in the number of viable AFB spores. The use of warm, dry air at the temperature of 160 - 180 °C destroys 100% of spores but only for 2 hours. The use of liquid paraffin at a temperature of 170 °C destroys AFB spores for 10 minutes (Dobbelaere et al., 2001). Earlier research by Japanese researchers found much lower concentrations of disinfectants that effectively neutralize AFB spores. This research was conducted in laboratory conditions and on culture media, without the presence of organic matter (Okayama et al., 1997).

These facts speak in favour of the importance of the method of decontamination of equipment and accessories in infected beehives. In Serbia, as a measure of disease control, the destruction of diseased colonies is used, where laboratory diagnostics is used to determine a positive finding for the presence of the causative AFB agent. The diseased colonies are closed, and the method of suffocation with the sulpfur strips is most commonly used. After that, complete beehives with honeycomb are burned, on the very location of the bee yard or in the immediate vicinity. The choice of the method of destroying the causative agent, the choice of disinfectant, as well as the concentration for the preparation of working solutions has a crucial role in the control of this disease. If it is known that the spores are extremely resistant to the commonly used concentrations of disinfectants, it is a question whether after the disinfection of the entire apiary the spores of the causative agent are destroyed and to what level. The fact that bee yards and hives are most often found on the ground, the effect of disinfectants on the surface of the earth is extremely limited to the surface layer and if used in recommended concentrations that definitely destroy spores, so the economic aspect of disinfection is questionable as well as the problems of environmental pollution by various chemical compounds.

To our knowledge, there is no published data about AFB genotypes in Serbia. Based on research conducted in Slovenia, in the period from 2017 to 2019, it was determined that 70.2% of the causative agents of AFB belong to the EPIC II genotype, and that 29.8% belong to the EPIC genotype EPIC I (Žugelj et al., 2021). It has been proposed that the fast killing phenotype allows nurse bees to remove infected larvae more efficiently (Rauch et al., 2009). In the colonies with good hygienic instinct, dead bee larvae will be expelled, and therefore that the common clinical symptoms of the disease in colony will be missing, as is the case with slow-killing genotypes. We can hypothesize that this finding could be connected with the reports of several veterinary inspectors, who reported difficulties in finding AFB clinical symptoms on larvae, because of spotty brood and missing dead or infected larvae under capes. Thus, the ERIC

II major prevalence and faster death of larvae in combination with the hygienic behaviour of bees can result in problematic recognition of clinical symptoms which, as a result, causes a delayed confirmation of AFB disease on the clinical level (Rauch et al., 2009). The moment when a beekeeper starts suspecting that there are diseased colonies in his bee hive is often crucial, because during this period, a beekeeper unintentionally spreads the pathogen over the apiary. In the case of migrating beehives, this poses an additional risk. One of the main reasons why many cases of AFB remain undiagnosed is that a large number of beekeepers do not recognize the disease or do not want to report it, out of the fear from the consequences, and because they try to disguise or solve it the problem on their own.

A crucial time for each of these variants is when the causative agent of AFB spreads to wider areas through contaminated bees from the diseased bee colony. Another important factor in the spread of AFB is the method of beekeeping, i.e. whether it is a stationary or mobile method. In Serbia, a large number of beekeepers is involved in mobile beekeeping. In early spring, some of the beekeepers decide to move their hives to oilseed rape crops in Vojvodina Province. The largest number of beekeepers move their hives from April to July to the Cer Mountain for acacia foraging and after that to the linden foraging on Fruška Gora Mountain. This is followed by sunflower foraging on the territory of Vojvodina Province. This migration of a large number of beekeepers and the concentration of a large number of bee colonies in the same locations is one of the extremely important factors in the spread of the pathogen throughout the country. Researchers in Slovenia used molecular epidemiology methods to determine the connection between certain genotypes of AFB pathogens in different localities in the country caused by migration of bee colonies (Žugelj et al., 2021).

Transmission of spores within or between bee colonies occurs by contaminated adult bees and honey or by interventions of the beekeeper (Genersch, 2010). AFB is not only horizontally transmitted between colonies, e.g. through diseased, weakened colonies being robbed out by other colonies and (Lindström et al., 2008) but also vertically through swarming of strong although infected colonies. One question, however, is still open in this context: Some researchers still address the following question is swarming really a vertical transmission route for *P. larvae* on colony level or is it a cure? (Fries et al., 2006).

In Serbia, after establishing a laboratory diagnosis of the presence of the causative agent of AFB in a beehive, the measures applied are closing and destroying the infected bee colonies. In order to calculate the compensation, a commission that assesses the level of damage needs to be formed, so the period

since the initial suspicion, laboratory diagnostics, and then the destruction of the colony and disinfection of apiaries can be quite long. During this period, a beekeeper may inadvertently transmit the causative agents within the apiary.

In Serbia, the treatment of bee colonies with antibiotics is not allowed, but since samples of antibiotics can be found in honey, it can be assumed that they are still used illegally (Kartalović et al., 2020). Antibiotics are not effective against the infectious spores, hence, they only suppress clinical symptoms and mask the disease but cannot cure AFB; chemical residues can persist in honey affecting its quality and safety for human consumption (Kartalović et al., 2020; Lodesani and Costa, 2005).

The ABF control measures that are applied, as mentioned, include the destruction of only bee colonies at infected bee yard when AFB has been confirmed by laboratory diagnostics. Other bee colonies are not destroyed, but quarantine is introduced, there is a ban on the relocation of bee colonies, the sale of queens, swarms, etc. (Anon, 1988). One of the greatest risks for the spread of AFB pathogens in Serbia is this measure, because a beekeeper will certainly spread the pathogens to other bee colonies when he works in the bee yard. There is a high risk of spreading the pathogen during this period.

In the chain of detecting clinical suspicion to AFB, the training of veterinarians for the tasks of this diagnostics is also an important factor, together with the readiness to cooperate with the veterinary inspection. Very few veterinarians are trained to inspect bee colonies, except for those veterinarians who are beekeepers themselves. Clinical examinations of bee colonies require a lot of physical effort and a long time. Veterinarians are not motivated to do this because the Veterinary Directorate has prescribed the price of a whole bee yard inspection, within the AFB active surveillance annul plan regardless of the number of hives at 2,000 Serbian dinars (approximately 16 €) and arriving at these places needs extra time. At the same time, it is much more profitable for a veterinarian to perform several other interventions that bring more money. If we add the fear of bees to this, inadequate protective equipment and problems with allergic reactions, it is clear why veterinarians hesitate to perform clinical examinations of bee colonies.

In Republic of Serbia, we can freely say that the largest number of beekeepers are those who do it as a hobby or because they need an additional source of income. Many beekeepers are pensioners. In the case of a beekeeper's illness or death, beehives are often left unattended and they perish. Such beehives are often the target for robber bees especially when the risk of spreading AFB, and other diseases is high. Old and abandoned apiaries and beekeeping equipment that can be found in various locations where people use it for various purposes

and can also be a source of AFB if they come from infected beehives, and if adequate decontamination was not performed.

CONCLUSION

The AFB occurs in Serbia every year and causes significant economic damage to beekeepers. In the period from 2019 to 2021, the disease has been occurring consecutively in several locations, which proves the fact that the undertaken measures are not effective enough in the control of this disease. The regulations are being applied have not changed since 1988 and they need to be revised, especially those regarding destruction of exclusively infected colonies, and not the entire apiary. Also, very precise instructions/procedures should be introduced regarding the decontamination of the equipment and tools used in infected beehives, which effectively destroy the cause of this disease. In addition to this, timely detection of diseased bee colonies is an important step in prevention of the risk of disease spreading. Continuous education of beekeepers, veterinarians and veterinary inspection in the field of diagnosis of this disease and raising awareness of the importance of early diagnosis of as many cases of this disease as possible are some of the key factors in successful AFB control. Further research on the determination of AFB genotypes and its spatial distribution in Serbia is needed.

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Author's Contribution:

PV - writing manuscript and maps production, BĐ - data collection and preparations for analysis, JPR and JP - data analyses, RR - writing manuscript, MŽB and SJ - critical revision of the manuscript.

Competing interest

The authors declare that they have no competing interests.

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HONEYBEE VIRUSES PRESENCE IN SERBIAN APIARIES: A REVIEW

Tamaš Petrović[1*], Dejan Vidanović[2], Diana Lupulović[1], Gospava Lazić[1], Sava Lazić[1]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia
[2] Veterinary Specialized Institute "Kraljevo", Kraljevo, Republic of Serbia

Abstract

The honey bee *Apis mellifera* is an important beneficial insect recognized by production of honeybee products, having an important role in agricultural production as a pollinator, and playing an equally crucial role in conserving the biodiversity in many natural ecosystems. According to available literature data, during the period of more than 30 years, dramatic losses of honey bee winter colonies have frequently been reported all over the world, which could have a direct influence on human food resources and can affect not only apiculture or agriculture, but also pose an anthropological threat. One among many reasons for global bees-decline phenomenon is the influence of many viruses on honeybees' health. Until today, at least 24, and even more viruses were detected in honeybees, and for many of them the pathogenicity and impact on honeybees' health still remain unknown. However, it is well known that some of these viruses like acute bee paralysis virus, chronic bee paralysis virus, deformed wing virus, black queen cell virus, sacbrood virus, Kashmir bee virus, Israeli acute paralysis virus, slow bee paralysis virus, *Varroa destructor* virus-1 and some others have direct or indirect influence on individual honeybee or on whole honeybees' colony health. In this paper, an overview of existing literature data on the presence, prevalence and characterization of honeybee viruses detected in honeybee colonies and apiaries from different regions in Serbia from first detection of their presence in 1986 till nowadays is presented and discussed.

Key words: honeybees, virus presence and prevalence, Serbia

[1*] Corresponding Author: tomy@niv.ns.ac.rs

PREGLED PRISUSTVA VIRUSA PČELA U PČELINJACIMA U SRBIJI

Tamaš Petrović[1], Dejan Vidanović[2], Diana Lupulović[1], Gospava Lazić[1], Sava Lazić[1]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija
[2] Veterinarski specijalistički institut “Kraljevo”, Kraljevo, Republika Srbija

Kratak sadržaj

Medonosna pčela *Apis mellifera* je važan korisni insekt, prepoznat po proizvodnji pčelinjih proizvoda, koji ima važnu ulogu u poljoprivrednoj proizvodnji kroz oprašivanje, a igra podjednako ključnu ulogu i u očuvanju biodiverziteta u mnogim prirodnim ekosistemima. Prema dostupnim literaturnim podacima, u poslednjih više od 30 godina, širom sveta se često prijavljuju dramatični gubici pčelinjih društva nakon prezimljavanja, što može direktno uticati na resurse hrane za čoveka i može uticati ne samo na pčelarstvo ili poljoprivredu, već ima i antropološke pretnje. Jedan od mnogih razloga za globalni fenomen opadanja broja pčela je uticaj mnogih virusa na zdravlje pčela. Do danas je kod pčela otkriveno 24, pa i više vrsta virusa, a za mnoge od njih još uvek nije poznata patogenost po zdravlje pčela. Međutim, dobro je poznato da neki od ovih virusa, poput virusa akutne paralize pčela, virusa hronične paralize pčela, virusa deformisanih krila, virusa crnog matičnjaka, virusa mešinastog legla, Kašmirskog virusa pčela, virusa izraelske akutne paralize pčela, virusa spore paralize pčela, *Varroe destructor* virusa-1 i neki drugi, imaju direktan ili indirektan uticaj na zdravlje pčele kao individue ili na zdravlje cele pčelinje zajednice. U ovom radu je dat i razmotren pregled postojećih literaturnih podataka o prisustvu, rasprostranjenosti i karakterizaciji virusa medonosnih pčela otkrivenih u pčelinjim društvima i pčelinjacima iz različitih regiona Srbije od prvog otkrivanja njihovog prisustva u Srbiji 1986. godine do danas.

Ključne reči: medonosna pčela, prisustvo i prevalenca virusa, Srbija

INTRODUCTION

The western honey bee *Apis mellifera* is an important beneficial insect recognized by production of honeybee products, which plays an essential role in

the ecology of natural environments and in agricultural production through pollination (Petrović et al., 2013; Ćirković et al., 2018; Chapman et al., 2019). Almost one-third of distinctive agricultural crops need bees' pollination (O'Neal et al., 2018). Pollination services are mandatory for the production of crops like fruits, nuts and fibres, but also the production of many other agricultural crops is significantly improved by pollination (Tantillo et al., 2015). Also, honeybees play an equally crucial role in conserving the biodiversity in many natural ecosystems. The value of bee pollination services is commonly measured in billions of dollars representing about 9.5% of the value of crops across the world (Tantillo et al., 2015). In United States, the value of honeybee pollination for agriculture has been estimated at more than 14.6 billion dollars (Morse and Calderone, 2000). According to FAO and the European Union, the value of pollination is 20 – 30 times higher than the value of honey production (Antúnez et al., 2012).

According to available literature data, in the last 30 years, dramatic honey bee winter colony losses have been reported frequently from different regions all over the world (Breeze et al., 2014). Pollinating insect declines have a direct influence on human food resources and can affect not only apiculture or agriculture but also has anthropological threats (Francis et al., 2013; McMenamin and Flenniken, 2018). Among reasons for global bees-decline phenomenon several factors could be appointed like pesticides, destruction of habitat, modern industries, intensive agriculture, parasites/pathogens, climate change, and inadequate food supply (Ullah et al., 2021). The honeybees' health is often compromised by different pathogens, such as the mites *Varroa destructor* and *Acarapis woodi*, the microsporidia *Nosema ceranae* and *Nosema apis*, the bacteria *Paenibacillus larvae* and *Melissococcus plutonius* and different viruses (Antúnez et al., 2012). Many researchers demonstrated that viral infections in honey bee colonies are considered a key risk for their health at both individual and colony level (Tantillo et al., 2015; Levin et al., 2019; Beaurepaire et al., 2020; Ullah et al., 2021).

Viruses are mostly the hidden enemies of honey bees as compared to other pathogens, because most of infections pass without clinical manifestation or characteristic disease signs (Ullah et al., 2021). During the last two decades, honeybee virus infections have been increasingly investigated and have emerged as one of several causes of the honeybee colony losses (Tantillo et al., 2015). However, viruses are probably the least understood part of honeybee pathology mainly because of the lack of information of the objective data about viral disease outbreaks (Toplak et al., 2012). During the last few years, our understanding of the diversity of viruses infecting bee species has grown dramatically, especially due to rapid improvements and increasing accessibil-

ity of next-generation sequencing approaches. So far, honey bees have been reported to be the host to at least 24 and even more (Beaurepaire et al., 2020) viruses, primarily positive-strand RNA viruses belonging to order *Picornavirales*, and in the families *Dicistroviridae* and *Iflaviridae*. These ones are able to infect the different developing stages of the honeybees, including eggs, larvae, pupae and adults (Allen and Ball, 1996; Tantillo et al., 2015; Ullah et al., 2021). Most of them cause unapparent infections without clinical signs but in certain cases may cause serious or lethal diseases (Allen and Ball, 1996; Antúnez et al., 2012; Tantillo et al., 2015; Ullah et al., 2021). Of those viruses infecting honeybees, nine viruses are considered the most common ones that are able to cause severe disease. Those viruses include acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV) from the family *Dicistroviridae*, deformed wing virus (DWV), sacbrood virus (SBV), slow bee paralysis virus (SBPV), and *Varroa destructor* virus-1 (VDV1, or DWV-B) from the family *Iflaviridae,* and chronic bee paralysis virus (CBPV) as taxonomically unsystematic virus (Chen and Siede, 2007; de Miranda et al., 2010; de Miranda and Genersch, 2010; Antúnez et al., 2012; Toplak et al., 2012; Petrović et al., 2013; Chagas et al., 2019, Ullah et al., 2021). In addition, from 2015 to 2018, sequencing studies identified sequences corresponding to positive single-stranded RNA viruses from several other families and genera including *Tymoviridae*, *Secoviridae*, *Nodaviridae*, and *Flaviviridae* families; the *Sobemovirus* and *Negevirus* genera; the new genus *Halictivirus*; and a Nora-like virus; then negative single-stranded RNA viruses from the families *Bunyaviridae* and *Orthomyxoviridae*; also viruses from the family *Rhabdoviridae*, double-stranded RNA viruses from families *Partitiviridae* and *Totiviridae* as well as double-stranded DNA viruses (*Apis mellifera* filamentous virus and *Osmida cornuta* nudivirus), and single-stranded DNA viruses from *Circoviridae* and *Parvoviridae* families (Grozinger and Flenniken, 2019). Many of these viruses are also found in plants and fungi, and further studies will be needed to describe the role of many newly detected viruses and their potential influence on bees' health.

Often, clinically visible symptoms of honey bee virus diseases are mostly associated with other infectious agents, such as the presence of microsporidia *Nosema apis* and strong infestations with *Varroa destructor* mites (Ullah et al., 2021). Several studies implicate that the combination of certain virus infections and *Varroa destructor* infestation represent a serious threat to honeybee health (Allen and Ball, 1996; Chen et al., 2004; de Miranda et al., 2010; Antúnez et al., 2012; Tantillo et al., 2015; Ullah et al., 2021). An established opinion among researchers is that *Varroa* in association with a range of hon-

eybee viruses is a significant factor in the losses of managed honeybee colonies seen globally. The spread of this mite to the Western honeybee - *Apis mellifera*, and its ability to act as a viral reservoir, incubator, activator and transmitter has resulted in levels of certain viruses that affect the survival of the colony (Tantillo et al., 2015).

Viruses spread in honeybees by two ways: vertical and horizontal transmission (de Miranda et al., 2012; Chagas et al., 2019; Beaurepaire et al., 2020). In vertical transmission route, viruses could spread from the infected queen by trans-ovarial transmission, through drones by trans-spermal transmission, or during their mating - known as venereal transmission to the offspring. In horizontal transmission route, the viruses spread amongst colony members of same age generation and between same and different hives or apiaries via oral or contact route (de Miranda et al., 2012, Chagas et al., 2019; Ullah et al., 2021). In addition to this direct transmission routes, some viruses, like DWV, mainly spread among honeybees via *Varroa destructor* mites that become infected from infected bees and behave as a carrier to spread viruses to healthy bees when they feed on them (vector-borne transmission) that represents an example of indirect transmission (Ullah et al., 2021).

In nature, BQCV, DWV, KBV, and SBV infect larvae and pupae as well as adult bees, while ABPV affects only adult bees (Chen et al., 2004). The first detected honeybee virus was SBV detected at the beginning of 20th century (1913) in USA (White, 1913), much earlier than many of human and other animal viruses. SBV affects larvae of honeybees and causes sacbrood disease. Affected larvae change from pearly white to grey and finally black. When affected larvae are carefully removed from their cells, they appear to be a sac filled with water. The adult bees develop a latent infection characterized only by a decreased life span, without acclaimed symptoms (Berènyi et al., 2006). This latent infection is very important for the transmission of SBV since virus is accumulated in the head and in the hypopharyngeal glands of infected nurse bees that are responsible for feeding the larvae (Tantillo et al., 2015). Also, a positive correlation was observed between the intensity of *V. destructor* prevalence and the presence of SBV in adult bee samples (Tentcheva et al., 2004). The frequencies of SBV infection were much higher during the spring period, when the brood season begins and large numbers of susceptible larvae and young adults are present (Berènyi et al., 2006)

BQCV was first detected in brown and black coloured queen larvae and prepupae but it also affects larvae and pupae of worker bees without causing signs (Antúnez et al., 2012). In honeybee colonies, BQCV is more prevalent in adult bees than in the pupae, although it clinically affects mainly developing queen larvae and pupae of the queen, representing the primary hosts of

the virus. These larvae acquire a pale yellow appearance and the symptoms are similar to those caused by SBV infection (Tentcheva et al., 2004; Tantillo et al., 2015). An important correlation was observed between the incidence of BQCV and *Nosema apis* in honeybee colonies with a peak of infection and infestation during spring and early summer (Allen and Ball, 1996; Tantillo et al., 2015).

DWV was first isolated in Japanese apiaries from adult honeybees with a particular deformity of wings (Bailey and Ball, 1991), and is one of the most widely distributed honeybee viruses around the globe (Tantillo et al., 2015). The presence and high prevalence of DWV is reported in all continents except in Oceania (Allen and Ball, 1996). DWV has been detected in more than 20 bee species, and there is evidence that it replicates in several of these species and causes damaging symptoms in two bumble bee species (i.e., *Bombus terrestris* and *Bombus pascuorum*), as well as in *A. mellifera* (Grozinger and Flenniken, 2019). Infection with DWV is typically associated with the presence of *V. destructor*, and results in wings deformation in honeybees. Also, it is one of the most studied viruses that affect honeybees due to its relation with colony losses induced by honeybee's mite *V. destructor* (Antúnez et al., 2012). There are several routes of DWV transmission that can influence its abundance and virulence. In bee colonies, DWV is transmitted vertically from queen or drone to the offspring, and horizontally via trophallaxis and shared food resources, but very often by *Varroa destructor* as their biological vector (Grozinger and Flenniken, 2019). DWV is able to infect all bee developmental stages from eggs to adults, even if it shows a higher replication in pupae. DWV is a virus with a low pathogenicity and is often responsible for latent infections that can appear in clinical form after a stressful situation such as high infestation with *Varroa destructor* and some others (Tantillo et al., 2015). DWV levels in bees parasitized by *Varroa* are significantly higher than levels of bees infected by other routes. Also, *Varroa* appear to benefit from this interaction because they produce more offspring while feeding on DWV-infected pupae (Grozinger and Flenniken, 2019)

CBPV was first isolated in 1963 from diseased honeybees (Bailey et al., 1963) and it is present at all continents. This virus can persist throughout the years as a subclinical infection, and the prevalence of the infection did not follow any seasonal pattern (Tentcheva et al., 2004; Tantillo et al., 2015). At low levels, infected colonies do not show clinical signs, but high level of those viruses induces high mortality rates. CBPV induces abnormal trembling of the wings and body of honeybees and sometimes black individuals crawling at the hive entrance can be detected. These are the main two clinical symptoms in bees infected with CBPV. The wings are partially spread or dislocated. In some

cases, the crawling bees can be in large numbers representing the clinically evident disease (Antúnez et al., 2012). CBPV was detected in queens and all their offspring at all developmental stages including eggs. Crowded condition of the colonies promotes the spread of the virus by direct contact of healthy bees with paralysed individuals, but vertical virus transmission is also possible. Interestingly, CBPV infections have never been related to *Varroa destructor* infestations and the virus has not been reported (Tantillo et al., 2015).

ABPV is a common infective agent of honeybees, frequently detected in apparently healthy colonies (Allen and Ball, 1996; Bakonyi et al., 2002; Tentcheva et al., 2004; Berényi et al., 2006). Clinical form of infection is sometimes activated and exacerbated by stressful environmental factors such as *Varroa* mite infestations, bacterial infections, presence of insecticides or some other stress factors. In addition, ABPV has been indicated as one of the major factors contributing to the mortality of honeybees infested with *V. destructor* and it was detected to be a primary cause of mortality in weakened colonies from many countries like ex-Yugoslavia, Germany, France, Hungary and USA (Bakonyi et al., 2002; Tantillo et al., 2015). Clinically evident infection of ABPV is characterized by rapidly progressing paralysis, including trembling, inability to fly and the gradual darkening and loss of hair from the thorax and abdomen, and rapid death of adults. ABPV could attack all life cycle stages of honeybees, but the pupae are the most favourable hosts for virus multiplication (Chen et al., 2004; de Miranda et al., 2012; Tantillo et al., 2015). ABPV could enter the colony by foodborne and venereal transmission; however *Varroa destructor* plays a crucial role in spreading of this virus both as a vector and as an activator of viral infection (Tantillo et al., 2015).

IAPV was first isolated in 2004 from Israeli apiaries, where it caused a significant mortality in honeybees (Tantillo et al., 2015). The virus was found to be present in every developmental stage of honey bee and its infection was observed almost in all tissues, but mostly existed in hypopharyngeal glands, alimentary canal and nervous system (de Miranda et al., 2012). IBPV is closely related to KBV and ABPV. Besides Israel, IBPV is widespread in Australia and USA. Like ABPV and many other bee viruses, IBPV usually persists in bee colonies at low titres without clinical symptoms. However, different stress factors influencing the weakening of the honeybee defences can exacerbated the IBPV infection that leads to death of the honeybees. Clinical symptoms of such infection are very similar to the ABPV infection with rapidly progressing paralysis, including trembling, inability to fly, gradual darkening and loss of hair of the thorax and abdomen resulting in massive deaths of adult bees (Tantillo et al., 2015).

KBV is endemically present in Australia and in the USA, and has been reported in Europe only rarely (Tentcheva et al., 2004; Berènyi et al., 2006). Like IBPV and ABPV, KBV persists at low titres in apparently healthy colonies until different stress factors activate the viral multiplication causing the clinical manifestation of disease and death of the colony. Different developing stages of bees could be hit by infection, but without clearly defined disease symptoms. The transmission of KBV in naturally infected colonies can occur via multiple routes including foodborne transmission, but also transmission by *Varroa destructor* as physical or true vector of this virus. Recently, KBV was proved to be highly important marker of Colony Collapse Disorder (CCD), i.e., rapid loss of the colony's adult bee population (Tantillo et al., 2015).

Slow bee paralysis virus (SBPV) primarily affects the forelegs (paralyses) of honey bees but can also be found in the head, salivary gland, mandibular and hypopharyngeal glands, crop, fat body, while present in the thorax, midgut, hindlegs and rectum in low quantity. It was correlated with colony collapse in England, but it is mostly less prevalent in other European apiaries (de Miranda et al., 2012).

The diagnosis of bee virus infections is difficult because honey bee viruses usually persist as unapparent infections and cause no clinical signs of disease, and because the fact that bee colonies can be simultaneously attacked by more than one virus as well as other pathogens. Multiple viral infections in bees have been reported by a number of authors (Anderson and Gibbs, 1988; Chen et al., 2004; Toplak et al., 2012; Petrović et al., 2013; Simeunović et al., 2014; Ćirković et al, 2018).

The increased losses of bee colonies have been also reported in Serbia during the last two decades. Because of the effects of viral infections on honeybee health and honeybee colonies losses, the aim of our study was to assess the presence and distribution of different honeybee viruses in Serbian apiaries. In this paper, the authors presented an overview of existing literature data on the presence, prevalence and characterization of honeybee viruses detected in honeybee colonies and apiaries from different regions in Serbia, from the first detection of their presence in 1986 till nowadays.

THE PRESENCE OF HONEY BEE VIRUSES IN SERBIAN APIARIES

By reviewing the available literature data only limited number of studies on investigating the presence of viruses in Serbian honeybee colonies and apiaries were found. The first detection of viruses in Serbian apiaries has been done by Kulinčević and co-workers even 35 years ago (Kulinčević et al., 1990).

In that study, samples of adult honeybees were collected from 2 apiaries near Belgrade in autumn 1986 and early spring 1987, and tested on virus presence by electron microscope and immunodiffusion test (with different bee virus antisera). The results of the study confirmed the presence of 4 bee viruses, being: ABPV, Cloudy Wing Virus (CWV), DWV (at that time known as J strain of Egypt bee virus) and BQCV in Serbian apiaries.

After a long time period without any published data on the presence of honey bee viruses in Serbia, the first surveillance study on viruses presence based on molecular diagnostic methods was done in apiaries in two districts in Vojvodina Province, northern part of Serbia (Petrović et al., 2013). In this study, 30 bee samples originating from 15 apiaries at 13 locations in districts of South Bačka (7) and Srem (6) in Serbia, sampled from June 2011 to May 2013, were tested for the presence of six honeybee viruses: ABPV, BQCV, CBPV, DWV, KBV and SBV. The sampled bee colonies were of different health status ranging from apparently clinically normal colonies to ill or dead bees associated with abnormal mortally or sudden colony losses. By using one-step reverse transcription-PCR (RT-PCR) method, the presence of bee virus was detected in all examined samples (100%). The most prevalent virus was BQCV, present in all 30 (100%) samples, followed by SBV, DWV, CBPV and ABPV detected in 21 (70%), 20 (67%), 18 (60%) and 17 (57%) samples, respectively. KBV was not detected in any of samples. Petrović et al. (2013) pointed on the difference between the obtained data in regard to the location (district) of the tested apiaries. Among 20 analysed honeybee samples from 9 apiaries at 7 locations in South Bačka District all 20 (100%) samples were positive on the presence of BQCV, 17 (85%) on the presence of CBPV, 16 (80%) on the presence of SBV, 11 (55%) on the presence of DWV and 10 (50%) samples on the presence of ABPV. The results on virus presence in 10 bee samples from 6 apiaries and 6 locations on the territory of Srem District were slightly different. The most prevalent viruses were BQCV in 100% (10/10) and DWV in 90% (9/10) tested samples, followed by ABPV found in 70% (7/10), SBV found in 40% (4/10) and CBPV found in only 1 out of 10 (10%) examined samples. Besides BQCV, the most prevalent bee virus found in South Bačka District was CBPV and in Srem District it was DWV. The authors also pointed out that this difference could not be seen in clinical manifestation of the disease on the field. The lack of differences in clinical manifestations might be attributed to the relatively small number of examined samples or insufficient anamnestic data obtained from the field. If such differences really exist and remained unnoticed, it might be influenced by some factors that were not identified at that time. Further on, the data obtained in this study showed that in most of the tested samples (96.67%),

more than one virus was identified. Only one analysed sample (3.4%) was infected with only one virus, 2 (6.7%) with two viruses, 14 (46.7%) with three viruses, 6 (20%) with four viruses, and 7 (23.3%) samples were found positive on five viruses simultaneously. Almost the half of the analysed samples had 3, and more than 40% of the analysed samples had 4 or 5 simultaneous viral infections. The severity of clinical manifestations with high bee losses was associated with higher amount of viruses detected in the samples (Petrović et al., 2013). The results obtained in this study indicated high prevalence of 5 out of 6 examined bee viruses in Serbian apiaries, and the great burden of surveyed apiaries caused by simultaneous and multiple viral infections corresponds with the data reported from other countries (Chen et al., 2004; Tentcheva et al., 2004; Berènyi et al., 2006; Antúnez et al., 2012; Toplak et al., 2012).

Simeunović et al. (2014) tested the presence of bee viruses DWV and ABPV by real-time RT-PCR method in 55 seemingly healthy colonies located at 11 apiaries (5 colonies per apiary) distributed in northern, southern, eastern, western and central part of the Republic of Serbia. The results revealed the presence of DWV at each sampling location – tested apiary, and ABPV in 10 out of 11 apiaries. Among the analysed samples, DWV was detected in 76.4% and ABPV in 61.8% of tested samples. From the geographic point of view, the highest frequency of DWV and ABPV (both viruses were found in 100% of samples) was established in the northern region of Serbia (Vojvodina Province) with sampling locations in Odžaci and Bačka Palanka as well as locations Valjevo (Western Serbia) and Kladovo (Eastern Serbia). Contrary to these findings, ABPV was not detected in any of the samples from the western most sampled location (Prijepolje). Moreover, simultaneous infections with DWV and ABPV were identified in 50.9% of samples (28/55 colonies). Single infections with DWV were found in 23.6% samples (13/55 colonies) and with ABPV in 9.1% samples (5/55 colonies). At least one virus was detected in 85.5% samples and in 85.45% (47/55) colonies. Only 14.5% (8/55) of sampled colonies were negative for the presence of tested viruses (Simeunović et al., 2014). The authors concluded that high incidence of DWV and ABPV positive samples found in clinically asymptomatic colonies can be the consequence of inefficient and delayed *Varroa* treatment since the role of this mite in the transmission and activation of honey bee viruses is well known.

Similar, yet more extensive research was done by Ćirkovic et al. (2018), who studied the prevalence DWV, CBPV, ABPV and SBV in colonies of different strength located in five regions of Serbia. In addition, the genetic relationship between nucleotide sequences of detected bee virus strains from Serbia and those published in NCBI GenBank from different parts of the world

was investigated. In total, 150 colonies from 32 apiaries (about 5 colonies per apiary) located in 5 administrative regions in Serbia (Vojvodina Province as Northern Serbia, Western Serbia, Central Serbia, Eastern Serbia and Southern Serbia) were sampled during autumn 2017. The sampling strategy was to sample two strong, one medium and two weak colonies from each apiary. The selected colonies were without visible clinical signs of any disease. Using a real-time RT-PCR analysis at least one virus was detected in 87.33% of tested colonies. The most prevalent virus was DWV found in 74% colonies, followed by ABPV, SBV and CBPV (identified in 49.30%, 24.00% and 6.70% colonies, respectively). Single virus infection was found in 28.67% colonies (DWV, ABPV, SBV and CBPV in 21.33%, 4.0%, 2.67% and 0.67% colonies, respectively). In more than a half of tested colonies (58.66%), simultaneous infection with more than one virus was detected. In 12.67% of tested colonies, not any of examined viruses was found. With regard to the situation across the regions, DWV had the highest prevalence in all regions (66.70 – 83.30%), while the least prevalent virus was CBPV (0 – 19%). Except for DWV, the prevalence of the remaining three viruses differed significantly between the regions. The ABPV was highly prevalent in Vojvodina Province and in Eastern Serbia, but with a low prevalence in Central Serbia. Also, SBV was highly prevalent in Vojvodina Province and mainly low-prevalent in other regions. The highest prevalence of CBPV was detected in Southern Serbia, than in Eastern Serbia, quite low prevalence (about 5%) was detected in Central and Western Serbia, and the virus was not detected in apiaries from Vojvodina Province. No significant differences were found in the prevalence of DWV, ABPV, SBV and CBPV infections between weak, medium and strong colonies (Ćirković et al., 2018). For genetic relationship and phylogenetic analysis partial coding sequences encoding a part of polyprotein gene of DWV, a capsid protein gene of ABPV, a part of RNA-dependent RNA polymerase (RdRp) gene of CBPV, and a part of polyprotein gene of SBV of detected virus strains in Serbia were used along with related sequences deposited in NCBI GenBank. The similarities found for 9 DWV strains from Serbia with deposited nucleotide sequences in NCBI GenBank were 99 to 98%. Some of the nucleotide sequences of Serbian DWV strains were highly similar to those from United Kingdom, but most of them were similar to virus strains from different countries, which is in line with the hypothesis of relatively recent evolutionary diversification of DWV and its worldwide distribution. The similarity between nucleotide sequence of ABPV strain from Serbia and those deposited in NCBI GenBank was between 93 and 97%. The Serbian ABPV strain was distant from those from north and western European countries, and the highest similarity was found with Hungarian

ABPV strains. This may be explained by the geographical vicinity and trade between beekeepers of the two countries. The Serbian SBV strains were mostly similar to those from different European countries and Russia, and more distinct from strains from other parts of the world. The similarity between nucleotide sequence of Serbian CBPV strain and other deposited in NCBI GenBank was only from 93 to 96% indicating probable distinct virus evolution in different geographic areas and the need for more studies that should be done to obtain clearer picture (Ćirković et al., 2018)

Milićević et al. (2018) tested 30 honey samples from Serbian apiaries originating from the local markets sampled from 12 different districts in central and northern Serbia, as well as 40 samples of both honeys and live and dead bees, from four apiaries without any visible health disorder, located in central Serbia. The samples were tested for the presence of BQCV, KBV, DWV, ABPV, SBV, and CBPV by multiplex RT-PCR. Out of all six examined viruses, only BQCV genome was found in 24 honey samples (80%) from the local markets. Also, BQCV was the only virus found both in bees and honey samples from tested apiaries. The virus was detected in three out of four apiaries, and the virus prevalence at a hive level was between 86.6 and 100%. The partial polyprotein coding region of detected BQCV strains were sequenced. The phylogenetic analysis showed that Serbian BQCV strains were similar to each other (98.5%), and mostly similar to some other European strains (86.4%) clustered by geographical origin. The high similarity observed between Serbian and Hungarian isolates that clustered together was found to be quite expected, due to the close trading and communication between two neighbouring countries, the same or similar habitat, and pasture. The most divergent virus strains were those from the USA (Milićević et al., 2018).

The testing of bees samples taken from diseased or dead honey bee colonies provided by beekeepers all over the Republic of Serbia to the Department of Biology, Faculty of Veterinary Medicine, on the presence of viruses in the five-year period (2014 - 2018) showed that the prevalence was between 73.12 and 87.16% for DWV, 61.54 and 81.45% for ABPV and between 58.82 and 64.22% for CBPV, depending of the year of testing (Stanimirović et al, 2019).

In addition to previously described studies, Tarić et al. (2019) investigated the differences between the presence of virus infections of the brood and adult bees depending of the used apiculture technology (conventional vs. traditional). There are certain regions in the Republic of Serbia where bees are still kept in a traditional way, in primitive hives made of wicker – so-called trmka hives. The study was conducted on the Pešter Plateau (1,059 km2), Raška District, southwest Serbia. Samples of bees from 144 asymptomatic honey bee colonies (120 kept in commercial hives and 24 colonies kept in traditional trmka

hives) originating from 18 apiaries (15 commercial apiaries and 3 apiaries with trmka hives) were tested for the presence of the ABPV, CBPV, DWV and SBV by molecular diagnostic methods. The SBV was detected in 96.67% samples from commercial colonies and in only 33.33% samples from traditional hives. In addition, the occurrence of viruses in adult bees was significantly higher in commercial colonies. The ABPV was found in 96.67%, and CBPV and DWV were detected in 100% of bee colonies for commercial purpose, contrary to the 33.33% colonies detected positive for ABPV, CBPV and DWV in traditional apiaries. Only one out of three apiaries with traditional hives was found positive for one or more viruses. The obtained data showed that in the brood and adult bees without clinical symptoms reared in a traditional way in primitive hives, the prevalence of all monitored viruses was up to 33.33%, which is within the limits of normal distribution of viruses in bee colonies in natural conditions. Moreover, traditional trmka hives provide significantly better conditions for maintenance of bee health and their resistance to pathogens (Tarić et al., 2019).

The results of all mentioned published studies are summarized in Table 1. With the exception of the first studies done by Kulinčević et al. (1990) applying serological test and the studies performed by Milićević et al. (2018) on honey samples or on small number of apiaries (4), in which some of bee viruses (CBPV and SBV or ABPV, CBPV, SBV and DWV) were not detected, in all other studies, all tested viruses (ABPV, CBPV, SBV, DWV and BQCV, except CWV) were detected at a high percentage. In these studies, ABPV was present in a range from 7.14 up to 90% of samples, CBPV from 3.3 up to 100%, SBV from 3.3 up to 96.67%, DWV from 3.3 up to 100% and BQCV from 50 up to 100% of tested honeybee samples. CWV presence was tested in only one study (Kulinčević et al., 1990) in small number of samples and found to be present in up to 20% of the honeybee samples. Only KBV was not detected in any of tested samples and studies in Serbia (Table 1).

Table 1: Presence and prevalence of honeybee viruses (ABPV, CBPV, SBV, DWV, BQCV, KBV and CWV) in examined honeybee (and honey) samples and apiaries from different regions of the Republic of Serbia from 1986 until 2019

Location	No samples / hivess (apiaries)	Year of testing	Presence / prevalence of honeybee viruses %							References
			ABPV	CBPV	SBV	DWV	BQCV	KBV	CWV	
Belgrade	5 (2)	1986	60.0	0	0	100	0	/	20.0	Kulinčević et al., 1990
Belgrade	14 (2)	1987	7.14	0	0	0	50	/	14.29	

Location	No samples / hivess (apiaries)	Year of testing	Presence / prevalence of honeybee viruses %							References
			ABPV	CBPV	SBV	DWV	BQCV	KBV	CWV	
South Bačka District	20 (9)	2011-2013	50.0	85.0	80.0	55.0	100	0	/	Petrović et al., 2013
Srem District	10 (6)	2011-2013	70.0	10.0	40.0	90.0	100	0	/	
Vojvodina province – Northern Serbia	10 (2)	2013	100	/	/	100	/	/	/	Simeunović et al., 2014
Western Serbia	15 (3)	2013	46.67	/	/	60.0	/	/	/	
Central Serbia	15 (3)	2013	26.67	/	/	73.33	/	/	/	
Eastern Serbia	10 (2)	2013	90.0	/	/	80.0	/	/	/	
Southern Serbia	5 (1)	2013	40.0	/	/	80.0	/	/	/	
Central & Northern Serbia -honey samples	30** honeys	2015 - 2016	0	0	0	0	80.0	0	/	Milićević et al., 2018
Central Serbia	40 (4)	2016-2017	0	0	0	0	80.0	0	/	
Vojvodina province – Northern Serbia	50 (10)*	2017	68.2	0	54.5	70.5	/	/	/	Ćirković et al., 2018
Western Serbia	30 (6)*	2017	46.7	23.3	6.7	66.7	/	/	/	
Central Serbia	30 (6)*	2017	16.7	3.3	3.3	83.3	/	/	/	
Eastern Serbia	25 (5)*	2017	56.0	12.0	4.0	80.0	/	/	/	
Southern Serbia	25 (5)*	2017	52.4	19.0	14.3	71.4	/	/	/	
Pešter Plateau, Raška District (commercial hives)	120 (15)	2017	83.33	100	96.67	100	/	/	/	Tarić et al., 2019
Pešter Plateau, Raška District (trmka hives)	24 (3)	2017	33.33	33.33	33.33	33.33	/	/	/	

Location	No samples / hivess (apiaries)	Year of testing	Presence / prevalence of honeybee viruses %							References
			ABPV	CBPV	SBV	DWV	BQCV	KBV	CWV	
Serbia (different regions)	195	2014	61.54	60.0	/	84.62	/	/	/	Stanimirović et al., 2019
Serbia (different regions)	221	2015	81.45	58.82	/	76.47	/	/	/	
Serbia (different regions)	200	2016	67.5	60,0	/	85.5	/	/	/	
Serbia (different regions)	186	2017	65.05	55.38	/	73.12	/	/	/	
Serbia (different regions)	218	2018	77.98	64.22	/	87.16	/	/	/	

/ – not done; * approximately 5 samples per apiary had been tested; ** honey samples from lokal markets

The results obtained in all afore mentioned studies in Serbia, revealed a quite high prevalence of the most important bee viruses on the whole territory of Serbia. The prevalence of DWV in Serbian apiaries is similar with the findings in Hungary (72%) (Forgách et al., 2008) and Slovenia (70%) (Toplak et al., 2012), and slightly lower than 97%, 91%, 97% and 100% found in France, Austria, England, Uruguay and Croatia, respectively (Tentcheva et al., 2004; Berènyi et al., 2006; Baker and Schroeder, 2008; Antúnez et al., 2006; Gajger et al., 2014).

The prevalence of CBPV in the Republic of Serbia obtained in the most of the studies is higher than that reported in other European and surrounding countries. CBPV was rarely detected in French (28%), Slovenian (18%), Croatian (10%), Austrian (9%), Danish (4%) and Turkish (1.8%) surveys (Tentcheva et al., 2004; Toplak et al., 2012; Gajger et al., 2014; Berènyi et al., 2006; Nielsen et al., 2008; Cagirgan and Yazici, 2021). However, the obtained prevalence of ABPV in Serbian apiaries (57%) is similar to the results reported in Slovenia (40%), Hungary (37%), France (58%) and Austria (68%) (Toplak et al., 2012; Forgách et al., 2008; Tentcheva et al., 2004; Berènyi et al., 2006), but higher than those reported in Croatia (10%), Denmark (11%) and Turkey (3.6%) (Gajger et al., 2014; Nielsen et al., 2008; Cagirgan and Yazici, 2021).

BQCV was reported as highly prevalent in studies that tested the presence of this virus in the Republic of Serbia. This virus infection has been reported to have a variable prevalence in different countries, with 1, 10, 18, 20, 30, 54, 83, 86 and 90% detection rates in Denmark, Spain, Turkey, Croatia, Hungary, Slovenia, France, Austria and Uruguay, respectively (Nielsen et al., 2008; Antúnez et al., 2012; Cagirgan and Yazici, 2021; Gajger et al., 2014; Berènyi et al., 2006; Forgách et al., 2008; Toplak et al., 2012; Tentcheva et al., 2004; Antúnez et al., 2006).

SBV was found as highly prevalent in apiaries in Vojvodina Province and in Raška District (Pešter Plateau) similarly to the results reported in Denmark, France, Croatia, Austria and Uruguay, where SBV was identified in 81, 86, 70, 48 and 100% samples, respectively (Nielsen et al., 2008, Tentcheva et al., 2004; Gajger et al., 2014; Berènyi et al., 2006; Antúnez et al., 2006). However, these values were much lower in central, eastern and south-eastern parts of Serbia similarly to the results obtained in Spain (1.1%), England (1.4%), Hungary (2%), Slovenia (8.3%), and Turkey (2.7%) (Antúnez et al., 2012; Baker and Schroeder, 2008; Forgách et al., 2008; Toplak et al., 2012; Cagirgan and Yazici, 2021).

In studies outside of Europe, that is, in different regions of China, in a study conducted in the period 2011 - 2012, BQCV was reported the prevalent virus (90 - 100%), followed by DWV detected in 40 – 100%, SBV in 0 – 100% and IAPV found in 0 - 60% of honeybee samples. CBPV and ABPV were detected only sporadically, and KBV was not detected at all (Ding et al., 2016). In different regions of Argentina, in a study conducted in the same period, DWV was found to be most prevalent ranging from 9 to 90%, followed by BQCV found sporadically in up to 60% of the honeybee samples. ABPV and CBPV were found only sporadically 0 - 40%, and SBV, KBV and IAPV were not detected at all (Ding et al., 2016).

CONCLUSION

The presence of bee viruses has been studied in the Republic of Serbia for more than 35 years, yet there are only a few studies performed and published mainly in the past 5 to 10 years. High prevalence of different bee viruses identified in Serbian apiaries in most of the studies may be partly explained by high density of bee colonies in geographic regions from which bee samples were collected. Also, the intensive and uncontrolled trade and transport of bee colonies, queens, or equipment could be one of the reasons for the transmission and spread of these viruses between apiaries. The tradition of beekeeping and intensive beekeeping practice in Serbia, as well as many different challenges

influencing the health of bee colonies impose the need for more comprehensive and continuous studies on virus presence and influence on bee health. Viruses, already present in apiaries in the Republic of Serbia, in different aspects of decreased immunity in bees, could become the ultimate extinction factors for bee colonies. The knowledge on virus's presence and prevalence in apiaries will influence the existing trading practices and better protection of healthy apiaries, and improve the management of infected apiaries in the Republic of Serbia.

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Author's Contribution:

TP – write the first draft of the manuscript and made substantial contributions to the basic idea, DV - contributions to the basic idea, DL and GL - were involved in drafting of the manuscript, DV and SL - critical revision of the manuscript.

Competing interest

The authors declare that they have no competing interests.

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CONTROL AND VIABILITY OF BEE NOSEMOSES

Igor Stojanov[1*], Radomir Ratajac[1], Jasna Prodanov Radulović[1], Jelena Petrović[1], Mirjana Bojanić Rašović[2], Ivan Pušić[1]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia
[2] Biotechnical Faculty, University of Montenegro, Podgorica, Montenegro

Abstract

Nosemosis is a bee disease practically present in all countries of the world. The control of the presence of *Nosema apis* and *Nosema ceranae* is of great importance. The consequences for bees will depend on the degree of infection of a bee colony. Weakening of bee colonies, reduction of production potentials, contamination with bee feces from hives, death of bees and / or their complete disappearance are some of the signs of the disease. The bee life physiology, which largely depends on the season, and regular control of the pathogen presence can determine the perspective of the disease. The consequences of reduction of the number of bee colonies are not only reflected in a smaller amount of bee products (honey, royal jelly, pollen, wax), but also in agricultural production in the form of reduced pollination. Domestic and international trade in bee products plays an important role in the transmission of *Nosema* spp. and other infectious and parasitic diseases of bees. Therefore, the control of the presence and viability of *Nosema* spp. in some bee products is important. Our analysis showed that the control of the presence of the cause of nosemosis in our conditions, without clearly defined legal regulations, is insufficient with regards to the number of beekeepers, bee colonies and hives. On the other hand, reducing the viability of *Nosema* spp. in different temperature conditions provides an opportunity for safe trade.

Key words: bees, nosemosis, control, viability

[1*] Corresponding Author: igor@niv.ns.ac.rs

KONTROLA I VIABILNOST UZROČNIKA NOZEMOZE PČELA

Igor Stojanov [1], Radomir Ratajac[1], Jasna Prodanov Radulović[1], Jelena Petrović[1], Mirjana Bojanić Rašović[2], Ivan Pušić[1]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija
[2] Biotehnički fakultet Univerziteta Crne Gore, Podgorica, Crna Gora

Kratak sadržaj

Nosemoza predstavlja bolest pčela praktično prisutnu u svim zemljama sveta. Kontrola prisustva *Nosema apis* i *Nosema ceranae* od velike je važnosti jer u zavisnosti, od pre svega, stepena zaraženosti društva zavisiće i posledice po pčele. Slabljenje pčelinjih zajednica, smanjenje proizvodnih mogućnosti, zaprljanost košnica pčelinjim fecesom, uginuće pčela i/ili njihovo potpuno nestajanje su neki od znakova oboljenja. Fiziologija života pčela koje najvećim delom zavisi od godišnja doba i redovna kontrola prisustva uzročnika mogu da odrede perspektivu bolesti. Posledice smanjenja broja pčelinjih društava ne ogledaju se samo u manjoj količini pčelinjih proizvoda (med, mleč, polen, vosak) već se njihov nedostatak direktno odražava i na poljoprivrednu proizvodnju smanjenjem oprašivanja. Domaća i međunarodna trgovina proizvodima od pčela igra važnu ulogu u prenošenju *Nosema* spp. i drugih infektivnih i parazitskih bolesti pčela. Zato je kontrola prisustva i preživljavanja *Nosema* spp. u nekim pčelinjim proizvodima važna. Naša analiza je pokazala da je kontrola prisustva uzročnika nozemoze u našim uslovima, bez jasno definisane zakonske regulative, mala u odnosu na broj pčelara, pčelinjih društava i košnica. Sa druge strane smanjenje mogućnosti preživljavanja *Nosema* spp. u različitim temperaturnim uslovima pruža mogućnost za bezbednu trgovinu.

Ključne reči: pčele, nozemoza, kontrola, vijabilnost

INTRODUCTION

Honeybees (*Apis mellifera*) are organisms that are very well adapted and able to survive in different climate, geographical and ambient living conditions. For these reasons, bees are capable of successful reproduction and development around the planet. Today, the importance of their presence is meas-

ured by economic results, directly, by the value and scope of bee products, and indirectly in agriculture by their role in pollination (Aizen and Harder, 2009; Bradbear, 2009). The presence of the causative agent of nosemosis directly leads to a decrease in the lifespan of bees, increased winter death, it affects the strength of bee colonies and their productivity (Botías et al., 2013). The possibility of relocating bee colonies and international trade has contributed, on the one hand, to better economic conditions for beekeepers, and on the other hand, it enabled bee products to reach the markets around the world. The trade of queens and bee products (honey, royal jelly, propolis, wax and bee venom), led to a risk of spreading various infectious and parasitic bee diseases. Any of the mentioned forms of trade in bee products can lead to the transmission of the following: the causative agent of American foulbrood of honey bees (*Paenibacillus larvae*), European foulbrood (*Melissococcus plutonis, Streptococcus pluton, Bacillus alvei, Achromobacter eurydice*, *Streptococcus faecalis, Bacillus laterosporus*), Nosemosis (*Nosema apis, Nosema ceranae*), Varroa (*Varoa destructor*), Ethinosis (*Aethina tumida*), Tropileleosis (*Tropilaelaps* spp.), Ascospherosis (*Ascosphaera apis*) and various viral diseases (Mutinelli, 2011).

This study will highlight the problem of nosemosis in beekeeping. Nosemosis is an infectious disease of the digestive tract of bees caused by a single-celled parasite, formerly classified as a protozoan, which today belongs to fungi (Microsporidia) (Adl et al., 2005). Nosema is an intracellular parasite that attacks the epithelial cells of the middle part of the bee's gut. Infection occurs by consuming contaminated food or water, sharing food between bees and during hive cleaning (Martın-Hernandez et al., 2018). The study will look into the data on the number of examined bee samples during the last 6 years (2015 - 2020), with the aim to draw attention to the importance of laboratory analyses. The data related to the sensitivity of the pathogens of nosemosis to different temperatures and the possibility of their viability in different temperature conditions will also be presented.

MATERIAL AND METHODS

During 6 years, a total of 95 bee samples were examined for the presence of the nosemosis. The samples that were analysed were mostly living bees - few of them were dead bees. The tested materials contained ≥ 50 bees per sample. If the bee samples were alive, they were placed on ice or in jars with cotton wool soaked in ether in order to anesthetize (MacInnis et al., 2020). After 10 minutes, the abdominal part of the bee carcass was cut off. A few millilitres of water or saline solution and 20 - 50 severed bees should be placed in the mortar. If

the total number of spores needs to be determined, it is necessary to add saline solution (or water) in the quantity of 1 millilitre per bee (Fries et al., 2013). The abdomen is macerated with a pestle. The contents of the abdomen, light or dark ochre in colour, which have accumulated at the bottom of the mortar are transferred to a glass slide. The water is added if the contents are too thick. Drops of abdominal contents of bees that are transferred to the slide are covered with a cover glass. Microscopy is performed at a magnification of x 400.

RESULTS AND DISCUSSION

Nosemosis is an infectious bee disease that can be caused by two types of nosema: *Nosema apis* type A nosemosis and *Nosema ceranae* type C nosemosis (Higes et al., 2010; Martın-Hernandez et al., 2018). In addition to these two species, the presence of *Nosema neumanni* was confirmed in Uganda (Chemurot et al., 2017), which, according to its characteristics and pathogenesis, in not significantly different from the two above mentioned ones. The differentiation between *N. apis* and *N. ceranae* is almost impossible without the application of molecular methods, despite the fact that there are some morphological differences between their spores (Ptaszynska et al., 2014; Pelin et al., 2015). In addition to these characteristics, these two types of nosemosis also differ in clinical picture and pathogenesis. Nosemosis caused by *Nosema apis* is characterized by the finding bee feces in the hive and at the entrance of a hive, a decrease in the amount of collected honey, weakened movement in and out of the hive, increased death rate during winter and reduced development in spring (Fries, 1993). In nosemosis caused by *N. ceranae*, the mentioned symptoms are mostly absent or weakly expressed, while the general weakness of the bee colony and the increase in mortality are intensified (van der Zee et al., 2014).

Examination of bees for the presence of the cause of nosemosis was connected with the other analysis of the samples, which were brought for the control of the presence of some bacterial or parasitic diseases: American foulbrood of honeybees, varroasis, chalkbrood (*Ascosphaera apis*) and stonebrood (*Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus phoenicis*). All the tests that were performed, were related to the clinical changes in the bee brood or the bees themselves, where the owners brought samples in relation to beekeeping practice and experience. Table 1 contains the data on testing bees for the presence of the causes of nosemosis in the period 2015 - 2020.

Table1. The results of bee samples testing for the causes of nosemosis in a period of 6 years

	2015	2016	2017	2018	2019	2020	Σ	+	-
South Bačka District									
Novi Sad	2	19	8	8	12	1	50	37	13
Beočin	2	2		1			5	4	1
Bečej	6		3			3	12	12	1
Bačka Palanka	1	2	2	2			7	6	1
Bački Petrovac		4	3				7	4	3
Bač			2				2	1	1
Titel		3					3	0	3
Temerin	1						1	1	0
Srem District									
Sr. Karlovci			1				1	0	1
Ruma					3		3	3	0
Inđija				1			1	1	1
Sr. Mitrovica				2			2	2	0
Central Serbia									
Kragujevac				1			1	1	0
Σ	**12**	**30**	**19**	**15**	**15**	**4**	**95**	**70**	**25**

During the examination, the samples from two epizootiological areas were analysed: South Bačka and Srem District. Table 2 shows the data of the Association of Beekeeping Organizations of Serbia (Savez Pčelarskih Organizacija Srbije - SPOS), which were obtained from the Veterinary Directorate of the Ministry of Agriculture in December 2018.

Table 2. Number of beekeepers, beehives and marked hives in Serbia in 2018

Bee holder district	Number of beekeepers	Number of apiaries	Number of marked beehives
South Bačka	1,203	1,233	64,694
Srem	760	782	45,411
Total in Serbia	25,830	27,158	1,295,545

The examination of bees for the presence of the causes of nosemosis was part of the Annual Monitoring program for Animal Diseases in the Republic of Serbia for determining the program of animal health protection measures (sub law document).

During these 6 years, the program of control and monitoring has not considered bee illness connected with *Nosema* spp..The program included control and monitoring only of the causative agents of American foulbrood of honeybees, varroasis, tropylelosis and ethinosis.

The examined samples exclusively arrived for laboratory analysis with clinical findings related to large number of deaths in apiaries or disappearances of broods, poor conditions of bee colonies characterized by weakness of broods with reduced number of bees, suspicion of American foulbrood of honeybees. Beside to the findings of nosemosis, other causes of bee diseases were diagnosed: *Paenibacillus larvae*, *Varroa destructor* and *Ascosphaera apis* in one case.

The examinations that were conducted were mostly related to the end of winter and the beginning of spring. This fact indicates that, due to the absence of mandatory control, beekeepers bring samples for analysis at the moment when clinical problems have appeared. It is mainly the weakness of bee colonies, reduced number of bees, the death of bees or their complete disappearance.

The data from the literature (Botías et al., 2013) show how important it is to control the presence of *Nosema* spp., and the period, i.e., the part of year when the examination is performed. The authors examined the effect of the drug (nozecid) used to control nosemosis during the autumn and the spring, as well as a different number of treatments. *Nosema* spp. was confirmed in all study groups and the control group, too. Depending on the number of treatments with selected nozecid, the presence of *Nosema* spp.*sp*. decreased from 100% to 20 - 30%. A special effect was in the period before winter when bee colony is burdened with a very small percentage of the presence of *Nosema* spp.*sp*. or not even detected.

The possibility of spreading nosemosis was proven by the presence of *Nosema* spp. in the large wax moth (*Galleria mellonella*) (Ozgor et al., 2017). It is known that the large wax moth attacks weak bee colonies exposed to various parasitic or infectious diseases or those that are affected by pesticides from the environment (Ellis et al., 2013). The authors confirmed the presence of *N. apis* and *N. ceranae* by genotypic and phenotypic methods. The question to consider is - What is the infectious dose necessary for bees to become infected with microsporidia *Nosema* spp. Previous research has indicated that the average infectious dose for *N. apis* is 94.3 spores per bee, while for *N. ceranae* it is slightly higher, 149 spores per bee (Fries, 1988). Recent research indicates that

1.28 spores per bee are sufficient to cause nosemosis (McGowan et al., 2016). After the spores enter the digestive tract, the nosema transmits theirs sporoplasm to the enterocytes through its filaments, after which they multiply. The passage of spores along the digestive tract can lead to new infections along the intestines or their excretion (Martın-Hernandez et al., 2018). Occurrence of empty spores of *Nosema* spp. during the analysis of bee samples may indicate autoinfection with nosema, which, then causes intense decay of bee enterocytes (Higes et al., 2007; Higes et al., 2009).

Control of bee nosemosis should include determining the degree of infection of bees within a colony. In this way, it can be determined whether the bees are overloaded with *Nosema* spp. and whether it poses a threat to bee colony health. This is especially important if the displayed data is analysed. In 6 years, a total of 95 bee samples were examined for the presence of the cause of nosemosis. The samples were taken from two epizootiological areas which together, according to SPOS data from 2018, have just over 100,000 marked hives. On the other hand, it is particularly important to point out that tests are necessary before wintering bees. The presence of *Nosema* spp., even to a lesser extent in the autumn, can lead to their significant multiplication during the winter, due to autoinfection and the inability to clean the bee colonies due wintering. To determine whether bees are overloaded with nosemosis spores, it is necessary to prepare 25 - 50 bee abdomens. For each bee, it is necessary to add 1 mL of water before maceration. From the obtained suspension, 10 μL should be added to each haemocytometer chamber. The chambers should be examined at magnification of x 400 and the observed spores should be counted. The obtained number of spores should be multiplied by 50,000 and that is the number of spores per bee (Fries et al., 2013).

There are some data showing that the causative agents of nosemosis, *N. apis* and *N. ceranae* are highly resistant to increased or decreased temperatures and humidity and that these two types of *Nosema* are not equally resistant to changes in temperature. *N. ceranae* show greater resistance to elevated temperatures while spores lose their ability to survive at -18 °C after one week and are significantly more sensitive in this respect compared to *N. apis*. Those are significantly sensitive, in respect to *N. apis* (Fenoy et al., 2009; Malone et al., 2001; Martín-Hernández et al., 2009). These characteristics of *Nosema* may be the reason for a greater presence of *N. apis* in Northern European regions while *N. ceranae* is more present in the South (Retschnig et al., 2017). The study of viability and infectivity of *N. ceranae* after different temperature treatments of honey and beeswax was analysed in the paper presented by MacInnis et al. (2020). The authors wanted to determine to what extent survival and in-

fectivity of *N. ceranae* exposed to different temperatures during a period of one week to one year can be reduced. Exposure of honey with *N. ceranae* spores to the temperatures of 33 °C, 20 °C, -12 °C and -20 °C showed that the viability of microsporidia decreased by more than 50% after 4 weeks at 33 °C, after 100 days at 20 °C while a slightly decreased viability to 80% was recorded at -12 °C, and viability remained unchanged at -20 °C. When it comes to wax samples, the chance of spore survival fell to less than 10% after a week of exposure to temperatures of 33 °C, -12 °C and -20 °C, while at 20 °C viability dropped to 50% after 42 days.

CONCLUSION

Bee control for the presence of *Nosema* spp. is important both for bee health and the yields provided by the bees. The damage caused by the development of diseases and the weakening of colonies by infected bees is directly related to the reduction of bee production. The presence of *Nosema* spp. is, it might be said, a "silent killer" of beehives, especially if not controlled.

The analysis carried out in this study shows that during 6 years, an average of 17.5 bee samples are examined in one year. Considering the number of registered beehives and marked hives, the number of examined samples is so low that it represents a statistical error. Examination of samples for the presence of *Nosema* spp. can also be carried out in beekeeping organizations. It may be a good idea to share the data obtained by these controls with similar organizations in different municipalities, especially if these are the areas where hives are moved to during the feed season. It would also be good to inform the institutions dealing with animal health about the conducted analyses.

The knowledge of sensitivity/resistance of *Nosema* spp. in relation to the temperature differences during the year (the seasons) and geographical characteristics, as well as of their survival potential and maintenance of their infectivity can contribute to reduction of harmful effects of these microorganisms.

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Author's Contribution:

IS, JP and MBR made contributions to conception and design of the article, they were involved in data collection and drafting the manuscript. IP, JPR, RR and IS contributed with data about bee colonies, present diseases, microbiological results and estimation of epizootiological status. They revised the manuscript critically and together with IS, JP, MBR and JPR prepared the final draft of the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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Note for Contributors

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Examples of references:

Articles in journals:

1. Stojanović D., Maličević Ž., Ašanin R. 2002. The use a new model for the investigation of sepsis. Acta Veterinaria, 52, 2/3, 125-131.
2. Chen J. and McClane B.A. 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infection and Immunity, 83, 2369–2381. doi:10.1128/IAI.03136-14.
3. Williams R.B. 2015. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology, 34, 159-180. doi: 10.1080/03079450500112195.
4. Bailey M.A., Macklin K.S., Krehling J.T. 2013. Use of a multiplex PCR for the detection of toxin-encoding genes *netB* and *tpeL* in strains of *Clostridium perfringens.* ISRN Veterinary Science, Article ID 865702, 1-4. doi:10.1155/2013/865702.

Books:

5. Ficken, M. D. and Wages, D. P. 1997. Necrotic enteritis in Diseases of Poultry, Eds. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, Iowa State University Press, Ames, Iowa, USA, 10th edition, ISBN xxx-xxx-xxxxx-xx-x.

Chapters in books:

6. Plumb J.A. and Hanson L.A. 2011. Sturgeon viruses. In *Health maintenance and principal microbial diseases of cultured fishes*. Eds. J.A. Plumb, L.A. Hanson, 3rd edition, Blackwell Publishing, 219-225.

Articles in proceedings:

7. Giangaspero A., Marangi M., Pati S., Cafiero M.A., Camarda C., Sparagano O.A.E. 2011. Investigating the presence of acaricide residues in laying hens naturally infected by the red mite *Dermanyssus gallinae*. In *Book of Abstracts*, The 12th Asian food conference 2011, BITEC Bangna, Bangkok, Thailand, 27.
8. Vidanović D., Petrović T., Šekler M., Debeljak Z., Vasković N., Matović K., Plavšić

B., Dmitrić M. 2018. Avian influenza in Serbia: epidemiological situation during 2016–2017. In *Programme and Abstract book*, 11th International Congress for Veterinary Virology, 12th Annual Meeting of EPIZONE, 27-30.08.2018, University of Veterinary Medicine Vienna, Vienna, Austria, 118 (p187).

9. Lazić G., Lazić S., Bugarski D., Grubač S., Lupulović D., Samojlović M., Petrović T. 2018. Human enteroviruses in river water and sewage in Vojvodina. In *Book of Abstracts*, International Scientific Conference "Green economy and environment protection", Belgrade, 23-25. April 2018, edited by Larisa Jovanović, Belgrade, Naučno stručno društvo za zaštitu životne sredine "ECOLOGICA", 95-96. ISBN 978-86-89061-11-6.

Lows and Regulations:

10. European Union. 2003. Commission Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official Journal of the European Union, L 268:29. **https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2003R1831:20100901:EN:PDF**

Citations with organisations as authors:

11. European Food Safety Authority. 2016. Peer review of the pesticide risk assessment of the active substance benzoic acid. EFSA Journal, 14(12):4657-n/a. **http://dx.doi.org/10.2903/j.efsa.2016.4657.**

Software:

12. Statistica (Data Analysis Software System). 2006. v.7.1., StatSoft, Inc., USA (**www.statsoft.com**).

Web Links:

13. OIE: Animal Diseases. Available at: **http://www.oie.int/en/animal-health-in-the-world/information-on-aquatic-and-terrestrial-animal-diseases/.** Accessed 07.08.2019.
14. European Centre for Disease Prevention and Control (ECDC). Historical data by year - West Nile fever seasonal surveillance. Available at: https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical Accessed 31.07.2019.

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