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# STURGEON DISEASES IN AQUACULTURE

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## Abstract

Sturgeon aquaculture is economically important in many countries, for both meat and caviar production. Sturgeon is the common name for 27 species of fish belonging to the family Acipenseridae. Among them, only the sterlet (*Acipenser ruthenus*) and the Siberian sturgeon (*Acipenser baerii*) completes the lifecycle in fresh water. In Serbia, in the last few years, aquaculture enterprises have shown more interest in farming these fish species. Also, the importance of sturgeon aquaculture grows due to the rapid decrease of wild populations caused by overfishing, water pollution and destruction of habitat. The development of sturgeon aquaculture activities has been accompanied by the disease outbreaks, and possibility of the emergence and rapid dissemination of several infectious disease agents may represent serious problem in sturgeon aquaculture. Several viral, bacterial, fungal and parasitic diseases have been reported worldwide. Due to the limited knowledge about epizootiology and disease control methods, infectious diseases may represent a major challenge in sturgeon aquaculture. Moreover, none of the diseases reported in sturgeon are regulated in the World Organization for Animal Health (OIE) or European Union (EU) legislations. Due to the increasing interest in sturgeon aquaculture in Serbia present study is focused on the most important pathogens that may represent a threat to sturgeon aquaculture in Serbia.

**Key words:** sturgeon, Acipenseridae, aquaculture, diseases

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# BOLESTI JESETRE U AKVAKULTURI

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## Kratak sadržaj

Akvakultura jesetre ima veliki ekonomski značaj u brojnim zemljama, kako za dobijanje mesa tako i za proizvodnju kavijara. Jesetra je uobičajeno ime za 27 vrsta riba koje pripadaju porodici Acipenseridae. Među njima, samo kečiga (*Acipenser ruthenus*) i sibirska jesetra (*Acipenser baerii*) provode ceo životni ciklus u slatkoj vodi. Poslednjih godina, u Srbiji postoji veća zainteresovanost za uzgoj ovih vrsta riba. Značaj akvakulture jesetre raste zbog brzog opadanja njihovog broja u prirodi, izazvanog prekomernim izlovom, zagađenjem voda i uništavanjem staništa. Razvoj akvakulture jesetre je praćen pojavom bolesti, a mogućnost pojave i brzog širenja uzročnika zaraznih bolesti može predstavljati ozbiljan problem. Više virusnih, bakterijskih, gljivičnih i parazitskih bolesti je uočeno širom sveta. Zbog ograničenih podataka o epizootiologiji i metodama kontrole bolesti, zarazne bolesti mogu predstavljati veliki izazov u akvakulturi jesetri. Šta više, nijedna od bolesti utvrđenih kod jesetri nije regulisana od strane Svetske organizacije za zdravlje životinja (OIE) niti zakonodavstva Evropske unije (EU). Zbog sve većeg interesovanja za akvakulturu jesetre u Srbiji, u ovom radu je ukazano na najvažnije patogene koji mogu predstavljati pretnju za zapate jesetre u akvakulturi.

**Ključne reči:** jesetra, *Acipenseridae*, akvakultura, bolesti

## INTRODUCTION

Sturgeon farming has become an important and rapidly expanding sector of aquaculture worldwide. In the last decade, the farming of sturgeon in Western Europe has increased exponentially. The annual world production is allocated among 4 species: White sturgeon (*Acipenser transmontanus*), Siberian

sturgeon (*Acipenser baerii*), Adriatic sturgeon (*Acipenser naccarii*) and Russian sturgeon (*A. gueldenstaedtii*). This applies to both the growing of fry and fingerlings for restocking natural reservoirs as well as the production of table sized fish and caviar for the market. Sturgeon farming has become increasingly important worldwide for the production of caviar and fish flesh, as well as for restoration programmes set up to save endangered wild populations (Bronzi and Rosenthal, 2014). Several species are farmed, among which the most desirable are Russian (otherwise known as Danube) (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons due to their short reproductive cycles and desired products (caviar and meat) (Williot et al., 2001). However, the success of sturgeon farming is heavily restricted due to the paucity of information related to sturgeon diseases and related control methods. It is believed that sturgeons are comparatively more resistant to fish diseases; nevertheless, many studies have shown that their diseases are idiopathic and involve different pathogens. Intensive culture exposes the fish to several sources of stress, such as high stock densities and manipulations that predispose animals to a number of infectious diseases associated with viral or bacterial pathogens (Georgiadis et al., 2000). As in the culture of other fish species, diseases are a principal limiting factor in sturgeon farming. Among them, viral diseases often cause major damage to the industry. Disease control in sturgeon farming is difficult due to lack of knowledge about disease epidemiology and control methods (Ciulli et al., 2016). As a result of more intensive sturgeon aquaculture, infectious diseases that affect the species have emerged.

## Viral Diseases

The intensive breeding of sturgeon has facilitated the emergence and the spread of diseases. To date, several specific viruses have been found in sturgeons. Almost all of them were discovered in sturgeons native to North America, both in the USA and Canada, as well as in Europe, where these fish species were introduced (Raverty et al., 2003, Kelley et al., 2005).Viruses of the *Herpesviridae* and *Iridoviridae* families are the major threats for the sturgeon aquaculture and presents the most reported causes of mortality outbreaks in sturgeons (Raverty et al., 2003; Shchelkunov et al., 2009; LaPatra et al., 2014). Among them, the white sturgeon (*A. transmontanus*) iridovirus (WSIV) and white sturgeon herpesvirus type 2 (WSHV-2, later renamed to Acipenserid herpesvirus 2, AciHV-2) are economically the most significant pathogens, causing up to 95% mortality in cultured young sturgeon (Watson et al., 1995, Georgiadis et al., 2001, Kelley et al., 2005).

## Herpesviral diseases

Herpesviral diseases in sturgeons are caused by three viruses, the Acipenserid herpesvirus-1 (AciHV-1), Acipenserid herpesvirus-2 (AciHV-2), and Siberian sturgeon herpesvirus (SbSHV) (Kurobe et al., 2008; Shchelkunov et al., 2009).

## Acipenserid herpesvirus 1 (AciHV-1) disease

Acipenserid herpesvirus 1 (AciHV-1), also known as a white sturgeon herpesvirus 1 (WSHV-1), was initially isolated in 1991, from cultured juvenile white sturgeon (*Acipenser transmontanus*), in California (USA) hatcheries. Mortality was result of the severe infection of the integument and oropharyngeal mucosa (Hedrick et al., 1991). Histopathologically, the virus causes epidermal lesions and diffused dermatitis. AciHV-1 appears to be less virulent under experimental conditions than is AciHV-2 (Plumb and Hanson, 2011).

## Acipenserid herpesvirus 2 (AciHV-2) disease

Acipenserid herpesvirus 2 (AciHV-2) also known as a white sturgeon herpesvirus 2 (WSHV-2), was first isolated from ovarian fluid of an adult sturgeon and was a cause of mortality in farmed juvenile white sturgeon in North America in the mid-1990s (Watson et al., 1995). Later, a closely related virus was isolated in Russia, suggesting that the Russian isolates may have originated from North America. Mortality in adult fish infected with AciHV-2 was generally less than 10%. Experimentally, the shovelnose and pallid sturgeon were susceptible to AciHV-2 but other species were refractive (Mao et al., 1999). White sturgeon herpesvirus-2 occurs in older sturgeon with small white blisters which develop into the open lesions on the body surface. These lesions are frequently infected with secondary bacteria and/or ectoprotozoal parasites. Internally, the stomach and intestines are filled with fluid but other tissues appear normal. Wild white sturgeon which was infected with AciHV-2 became listless and stopped eating. Current management strategies for controlling AciHV-1 and AciHV-2 are avoidance of the agent and inspection of potential carrier fish via cell culture assay. Sturgeon infected with AciHV-2 could be prophylactically treated with salt and parasiticides to reduce secondary infections in open ulcers.

## Siberian sturgeon herpesvirus (SbSHV) disease

Siberian sturgeon herpesvirus (SbSHV) was isolated in Russia for the first time in 2006 from moribund Siberian sturgeon (*Acipenser baerii*) and bester (beluga *Huso huso* × sterlet *Acipenser ruthenus* hybrid) fingerlings during acute outbreak of disease on a fish farm (Shchelkunov et al., 2009).The infection has been found widespread in cultured sturgeon species in Russia. It is the cause of an acute necrohaemorrhagic skin syndrome complicated by secondary opportunistic infections (fungal, myxobacterial or protozoan) (Shchelkunov et al., 2009). Based on sequence analysis of the viral genome, it is determined that the SbSHV is a potential member of the genus Ictalurivirus within the family *Alloherpesviridae* under the order *Herpesvirales* (Doszpoly and Shchelkunov, 2010). Two Russian types of SbSHV (I and II) were isolated, and they differ from each other in four principal marker traits and each of the two has close genetic relationship with one or another strain of North American AciHV-2 species. It was hypothesized that the Russian type I and type II SbSHV may represent two different strains or genotypes of the Acipenserid herpesvirus 2 species (Doszpoly and Shchelkunov, 2010).

## Iridoviral diseases

Iridoviruses have been associated with severe disease and economic loss in fish with more than 50% mortality. The first iridovirus associated with mortality outbreaks of sturgeon was the white sturgeon iridovirus (WSIV) (Hedrick et al., 1990). Other iridolike viruses have been detected in different sturgeon species in North America and Europe, namely white sturgeon iridovirus (WSIV) (Hedrick et al., 1990, Raverty et al., 2003), Missouri River sturgeon iridovirus (MRSIV) (Kurobe et al., 2010, 2011), shortnose sturgeon virus (SNSV) (LaPatra et al., 2014), British Columbia white sturgeon virus (BCWSV), Namao virus (NV) (Clouthier et al., 2013), and Russian sturgeon iridovirus (Adkison et al., 1998). They can cause a lethal disease of the integumentary system in infected sturgeon, resulting in > 90% mortality within captive populations. In other instances, they are associated with a chronic debilitating wasting syndrome, resulting in severely impaired growth and reduced survival of fry and fingerlings (Clouthier et al., 2018). In total, nine species of sturgeon in the genera *Acipenser* or *Scaphirhynchus* of the family *Acipenseridae* were found susceptible to one or another virus. Outbreaks of virus disease are associated with stress factors such as rearing density, handling, fluctuations in water temperature, levels, and flow rates (Watson et al., 1998, Georgiadis et al., 2000, 2001). The phylogenetic

studies revealed that these viruses are only distantly related to Iridoviridae, and are included in a group of sturgeon nucleo-cytoplasmic large DNA viruses (NCLDVs). They do not form part of any currently recognized virus genera or family but do belong to the order *Megavirales* (Colson et al., 2013). NCLDV group has not formally been adopted by the International Committee for the Taxonomy of Viruses (Clouthier et al., 2015). The sturgeon NCLDVs are present in hatchery-reared and wild sturgeon found across North America and northern Europe (Clouthier et al., 2015), and pose a potential disease risk for strategies designed to aid the recovery of threatened and endangered sturgeon populations worldwide (Clouthier et al., 2018). Data on sturgeon iridolike viruses in Europe are limited despite the increasing importance of sturgeon aquaculture. Iridovirus-like infection has been reported in Northern Europe in Russian sturgeon (*Acipenser gueldenstaedtii*) associated with mortality (Adkison et al., 1998). NCLDVs outbreaks are frequently reported by sturgeon farmers, but only few thorough investigations have been conducted for these outbreaks. Sturgeon NCLDVs appear to be endemic in populations of *Acipenseridae* found throughout North America (Clouthier et al., 2015). At present, an accurate picture of the geographical distribution of sturgeon NCLDV infection in Europe is not available. Also, in 2009, frog iridovirus type 3 (FIV3), a well-known ranavirus provoked an outbreak on pallid sturgeon (*Scaphirhynchus albus*) in an American hatchery (Waltzek et al., 2014).

Recently, *Acipenser iridovirus* European (AcIV-E) has been detected in sturgeon populations in Europe. It appears that this virus is closely related to North American sturgeon iridoviruses, in particular with the white sturgeon iridovirus (WSIV) and the Namao virus (NV) (Axen et al., 2018). AcIV-E was detected and associated with clinical disease in different sturgeon species (*Acipenser baerii*, *Acipenser gueldenstaedtii*, *Acipenser naccari*, and *Huso huso*). Higher mortality due to AcIV-E was recorded in Russian sturgeon compared to Siberian sturgeon - 90% vs 50% - suggesting a lower susceptibility to the virus in Siberian sturgeon (Bigarré et al., 2017). Much important information, e.g., pathogenesis, transmission routes, and epidemiology of these viruses, is lacking, making virus and disease control extremely difficult (Axen et al., 2018).

## *Other sturgeon virus diseases*

### *White sturgeon adenovirus (WSAV) disease*

WSAV was identified in diseased juvenile white sturgeon between 1984 and 1986 (Hedrick et al., 1985) but the disease has not evolved into a serious

health problem, and there has not been any report on WSAV since then.

### *Infectious hematopoietic necrosis virus (IHN)*

Sturgeon are often cultured on facilities that contain other fish species. LaPatra et al. (1995) demonstrated that white sturgeon fry were susceptible to IHNV and that white sturgeons cultured with IHNV-infected rainbow trout had neutralizing antibodies to the virus.

### *Viral nervous necrosis (VNN)*

Betanodavirus within the family *Nodaviridae* is the etiological agent of viral nervous necrosis (VNN, also known as viral encephalopathy and retinopathy or VER). The virus infects a large range of host species in more than 50 species of marine and freshwater fish worldwide. Athanassopoulou et al. (2003) reported on the presence of a betanodavirus that infected sturgeon (*Acipenser gueldestaedi*) in fresh water causing disease with neurological signs.

### *Spring viremia of carp (SVC)*

Vicenova et al. (2011) reported isolation of the SVC virus in sturgeon with the clinical signs of the disease, which included internal haemorrhages, considerably enlarged bright-red spleen, and greyish-yellow liver showing tiny red spots. The sturgeon isolate was genetically identical to the isolate from clinically healthy koi carp collected from the same aquaculture site.

### *Infectious pancreatic necrosis (IPN)*

Little scientific information is available to assess whether sturgeon can become infected and develop into potential carriers of IPNV. Only the report from France indicated that IPNV was isolated from 3% (1 of 34) of a group of Siberian Sturgeon *Acipenser baerii* (Nougayrede, 1988).

### *Aquareoviruses*

Aquareoviruses are serious pathogens of aquatic animals. Reovirus particles were observed in diseased Chinese sturgeons, suggesting the existence of primary viral illness (Zhang and Gui, 2012).

### *Cyprinid herpesvirus-3 (CyHV-3)*

According to Pospichal et al. (2016) the hybrid between sterlet and beluga seems to be susceptible to cyprinid herpesvirus 3, but the authors could not prove that they can transfer this virus to naïve koi. Also, Kempter et al. (2009) reported that Atlantic sturgeon and Russian sturgeon are susceptible to CyHV-3.

### *Bacterial diseases*

Few mortality outbreaks have been reported in sturgeon associated with primary bacterial pathogens such as the case of lactococcosis in hybrid sturgeon, Bester (*Huso huso* x *Acipenser ruthenus*) (Chen et al., 2012). However, the isolation of bacteria from sturgeon was often reported as a secondary infection or as a consequence of severe stress and high stock densities. Motile Aeromonas infection (MAI) is one of the most common infection in sturgeon (Santi et al., 2019). MAI is usually associated with viral diseases in surviving sturgeon. Motile Aeromonas species have been isolated from sturgeon, generally as a consequence of severe stress and high stock densities or opportunistic, secondary to a primary viral infection. A motile aeromonas septicaemia caused by *Aeromonas hydrophila* frequently induced considerable losses in Persian sturgeon (*Acipenser persicus*) fingerlings in northern Iran (Soltani and Kalbassi, 2001) and also in the Harrison river sturgeon in Canada (Raverty and Nikl, 1999). *Aeromonas hydrophila* as a pathogenic agent has been isolated from Amur sturgeon (*Acipenser schrenckii*) in China (Meng et al., 2011). *Aeromonas hydrophila* and *Aeromonas veronii* were isolated during the outbreak of a disease characterized by haemorrhagic ascites and intestinal and renal haemorrhaging in cultured Chinese sturgeon (Di et al., 2018). In Turkey, *Aeromonas hydrophila* was detected in Russian sturgeon (*Acipenser gueldenstaedtii*) as the cause of bacterial haemorrhagic septicaemia and high rate of mortality (Timur et al., 2010). *Aeromonas veronii* was identified as a pathogen and cause of mortality of Siberian sturgeon (*Acipenser baerii*) (Ma et al., 2009). *Aeromonas veronii* was isolated from reared sturgeons in Iran (Gholamhosseini et al., 2018). *Aeromonas sobria* was isolated from *Acipenser gueldenstaedtii* and *Acipenser baerii* in Turkey (Kayis et al., 2017). *Pseudomonas* spp., particularly *Pseudomonas fluorescens*, are common worldwide and found mainly in cold freshwater. The infection was reported in *Acipenser baerii* by Brunetti et al. (2006) and in *Acipenser gueldenstaedtii* by Kayis et al. (2017). The pathogen *Pseudomonas alcaligenes* has been identified in Chinese sturgeon (Xu et al. 2015). *Streptococcus iniae* was isolated from liver, kidney and spleen of the dy-

ing sturgeons with clinical symptoms during an episode of continuous mortality of cultured hybrid sturgeons occurred on a farm in China in 2012 (Wang et al., 2014). *Yersinia ruckeri* was found responsible for 10% mortalities in cultured sturgeon (*Acipenser baerii*) in France (Vuillaume et al., 1987).

*Flavobacterium johnsoniae* was isolated from the diseased farmed young sturgeons (3-4 g) in Russia (Bauer et al., 2002). *Flavobacterium johnsoniae* and *Flavobacterium hydatis* isolates were associated with disease in cultured Russian sturgeon (*Acipenser gueldenstaedtii*) in Turkey (Karatas et al., 2010, Timur et al., 2010).

An outbreak of *Pseudomonas fluorescens* was reported in young (10 g in size) farmed Siberian sturgeon (*Acipenser baerii*) with high mortality (40%) in northern Italy (Brunetti et al., 2006).

Outbreaks of mycobacteriosis in reared sturgeons, including Chinese sturgeon (*Acipenser sinensis*), Russian sturgeon (*Acipenser gueldenstaedtii*) and Amur sturgeon (*Acipenser schrencki*), were identified (Zhang et al., 2017, Antuofermo et al., 2014, Righetti et al., 2014).

Several *Acinetobacter* isolates were detected in both *Acipenser gueldenstaedtii* and *Acipenser baerii* involved in the mortality outbreaks caused by NCLDV (Ciuli et al., 2016).

Lesions in *Acipenser gueldenstaedtii* and *Acipenser baerii* have been described during natural and experimental infections with *Acinetobacter johnsonii* and *Acinetobacter baumannii* (Kozinska et al., 2014).

*Streptococcus dysgalactiae*, a Gram-positive bacterium, has been isolated from *Acipenser schrenckii* in China. Haemorrhages, abdominal swelling and ascites have also been reported in cultured sturgeons (Yang and Li, 2009).

Also, *Yersinia ruckeri*, *Flavobacterium columnare*, *Flavobacterium psychorphilum* and *Renibacterium salmoninarum* have been isolated from sturgeons reared in recirculating aquaculture system (Pelkola et al., 2012).

### *Parasitic diseases*

Most common parasitic diseases are those in reared young sturgeons caused by ciliates of the genus *Trichodina* and other genera of the family *Urceolariidae*. Rarely occurs infections caused by *Ichthyophthirius multifilis* and *Chilodonella cyprini* (Bauer et al., 2002). Helminthic diseases of reared sturgeons are very rare in fish farms, although representatives of several species of *Monogenea*, *Trematodes*, *Cestodes* and *Nematodes* are sometimes found in reared sturgeons (Bauer et al., 2002).

### *Other diseases*

Fungal diseases caused by Saprolegniaceae are of great importance, especially during incubation of sturgeon eggs. Mortality of eggs during this period sometimes reaches 70-90%. Thirteen species of pathogens have been found, including *Saprolegnia* (seven species), *Achlia* (two species), *Aphanomyces* (one species), *Dactyunus* (two species) and *Zeptolognia* (one species) (Bauer et al., 2002). Jalilpoor et al. (2006) also isolated *Penicillium spp*., *Fusarium spp*., *Mucor spp.* and *Saprolegnia sp.* from the eggs of *Acipenser persicus*.

## CONCLUSIONS

The increasing importance of sturgeon farming and international trade in all species of sturgeon worldwide has increased the risk of disseminating specific pathogens and emphasizes the need for adequate diagnostics to prevent the spread of these pathogens. Most infections in natural conditions result in unapparent or mild disease, yet various pathogens can be highly pathogenic in sturgeon aquaculture where transfer of pathogens is facilitated by high densities of naïve hosts. As sturgeon is increasingly being farmed, a better understanding of the pathogens infecting this species is crucial to the development of a sustainable industry.

The majority of disease conditions on sturgeon farms could be significantly reduced if proper attention is paid to good husbandry and the maintenance of optimal environmental conditions, especially water quality.

In conclusion, the wide diversity of pathogens and the numerous movements of live fish and fish products have likely strongly contributed to the spread of infectious agents for years. The transfers of live material need to be better controlled in the future with an aim of improving fish health and hence their commercial and ecological value. Considering the potential of various infectious pathogens to cause severe disease in an aquaculture setting, additional studies are needed to increase the knowledge on the epidemiology of sturgeon diseases. This will help in controlling infections of managed stocks reared for food and also in conservation programs.

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# DID INTENSIVE FLOODS INFLUENCE HIGHER INCIDENCE RATE OF THE WEST NILE VIRUS IN THE POPULATION EXPOSED TO FLOODING IN THE REPUBLIC OF SRPSKA IN 2014?

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## Abstract

Climate change is definitely one of the greatest challenges of human development in the 21st century. Climate change is expected to increase the risk of communicable diseases in Europe. This impact will depend not only on local climatic conditions, but on other factors, such as current infrastructure, public health services, biodiversity specificity, etc. The population in Bosnia and Herzegovina, Croatia and Serbia has been severely affected by the floods caused by cyclone Tamara in 2014. The basic mode of transmission of the disease caused by the West Nile virus is the bite of the infected mosquito. The West Nile virus is not transmitted among humans through contact, nor can it be transmitted from infected birds to humans without mosquito bite. The aim of the study was to analyze and present the trend of this disease in the period 2014-2018 and to show the connection between the spatial occurrence of cases and location of the flooded area in 2014 in the Republic of Srpska. Using the descriptive method, the demographic data of the patients were analysed, the most common clinical form of the disease and the incidence of the disease in the period 2014-2018 was

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analysed. The cases were mapped and we analysed the connection of the case and location that was flooded. The incidence ranged from 0.79 in 2014 to 0.43 in 2018. Patients were of all age groups and both sexes, most commonly cases from rural areas (78%). The most common clinical form of the disease is shown, which were symptoms of the central nervous system infection. Out of the total number of patients, 94% were from flooded areas. All reported cases have been diagnosed at the Institute of Microbiology at the University Clinical Center of Republika Srpska and were reported as probable cases in accordance with the international case definition of communicable diseases. However, it is crucial to implement internationally endorsed procedures as a clinical alghoritm for the confirmation of the case in accordance with the laboratory criteria for the case definition. The occurrence, frequency and spatial distribution of cases indicates a possible connection with the floods in 2014.

**Key words**: West Nile Fever, Floods, Vector Diseases

# DA LI SU INTENZIVNE POPLAVE UTICALE NE VEĆU INCIDENCU VIRUSA ZAPADNOG NILA U POPULACIJI IZLOŽENOJ POPLAVAMA U REPUBLICI SRPSKOJ U 2014 GODINI?

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## Kratak sadržaj

Klimatske promjene su definitivno jedan od najvećih izazova humanog razvoja u 21. vijeku. Očekuje se da će klimatske promjene povećati rizik

od zaraznih bolesti u Evropi. Ovaj uticaj će zavisiti ne samo od lokalnih klimatskih uslova, nego i od drugih faktora, kao što su trenutna infrastruktura, usluge u sistemu javnog zdravlja, specifičnost biološke raznolikosti itd. Stanovništvo u Bosni i Hercegovini, Hrvatskoj i Srbiji je bilo teško pogođeno poplavama koje je izazvao ciklon Tamara 2014. godine. Osnovni način prenošenja bolesti Zapadnog Nila je širenje virusa ubodom zaraženog komarca. Virus Zapadnog Nila se ne prenosi među ljudima putem kontakta, niti se može prenijeti sa zaraženih ptica na ljude bez uboda komarca. Cilj studije je bio analizirati i prikazati kretanje ove bolesti u periodu 2014-2018. godina i prikazati moguću povezanost prostorne pojave slučajeva oboljenja sa poplavljenim područjem 2014. godine u Republici Srpskoj. Korišćenjem deskriptivnog metoda, analizirani su demografski podaci obolelih, a prikazana najčešća klinička forma bolesti i incidencija kretanja groznice zapadnog Nila u periodu 2014 – 2018. Slučajevi su mapirani i analizirana je povezanost lokacije slučaja sa područjem koje je bilo pogođeno poplavama. Incidencija se kretala od 0,79 u 2014. godini do 0,43 u 2018. godini. Pacijenti su bili svih starosnih grupa i oba pola, a najčešće su to bili slučajevi sa ruralnih područja (78%). Prikazana je i najčešća klinička forma obolelih, a to su simptomi infekcije i poremećaja centralnog nervnog sistema. Od ukupnog broja obolelih, njih 94% su sa poplavljenih područja. Svi prijavljeni slučajevi su dijagnostikovani u Zavodu za mikrobiologiju Univerzitetskog kliničkog centra Republike Srpske i vode se kao vjerovatni slučajevi u skladu sa međunarodnom definicijom slučajeva zaraznih bolesti. Međutim, ključno je sprovesti međunarodno usvojene procedure kao klinički algoritam za potvrdu slučaja u skladu sa laboratorijskim kriterijima za definiciju slučaja. Pojava, učestalost i prostorna raspodjela slučajeva ukazuje na moguću povezanost sa poplavama 2014.godine.

**Ključne riječi**: groznica zapadnog Nila, poplave, vektorske bolesti

## INTRODUCTION

A recent study by the European Center for Disease Prevention and Control has found that vector-borne diseases are one of the eight major communicable threats for Europe, along with the emergence and spread of resistance to antibiotics or pandemic influenza (Semenza et al., 2018). Climate changes affect the survival and transmission of arthropod vectors, as well as the rates of development of pathogens that are transmitted by the vectors. There was a significant correlation between mean temperature, precipitation and mosquito density (Lee et al., 2013). This resulted in the seasonal epidemics of West

Nile fever in the countries across the Europe for which there is no effective control measure such as vaccine in humans. Even the availability of an equine vaccine did not prove to be a solution because the coverage was low, probably due to sporadic epidemics and the relatively high cost of vaccine for the owners (Findlater et al., 2018). Ambient temperature is one of the most important abiotic factors affecting the life of the insect. Mosquitoes, such as *Aedes aegypti* and *Aedes albopictus*, transmit many pathogens, including Denge, Chikungunye and Zika viruses. The spread of these diseases has become the main global health issue and it is predicted that the climate change will affect the distribution of mosquitoes, which will enable these insects to introduce new pathogens into sensitive populations (Reinhold et al., 2018). Due to the combination of anthropogenic changes, including effects on the global climate and migration of wild animals, there is strong evidence that the regions with a moderate climate have repetitive occurrence of mosquito-borne diseases, but also the emergence of those diseases that have not previously been detected through surveillance.

In Europe, the re-emergence of West Nile virus (WNV) and Usutu virus is associated with the bird migration from Africa, while the autochthonous transmission of Chikungunya and Denga viruses is fuelled by a combination of invasive mosquitoes and rapid transcontinental travel by infected people (Johnson et al., 2018). Arboviruses are a diverse group of viruses that are transmitted by the vectors, many of which cause significant human morbidity and mortality. Continuous geographical expansion of the Dengue virus, along with explosive epidemics of the WNV, Chikungunya and Zika, served as a reminder that a new epidemics can occur at any time from this diversity. A clearer understanding of the mechanisms that trigger these dramatic changes in the transfer cycles from host to host that result in a significant exposure of the human population will help us prepare for the next epidemic/pandemic (Young, 2018). Arboviruses such as WNV, Zika, Chikungunya, Dengue and Yellow fever have become very significant global pathogens through unexpected, explosive epidemics (Rückert et al., 2018).

A constant occurrence is evident during summer months in Europe (from July to September) when higher ambient temperatures favor the activity of mosquitoes, while winter months (December to February) generally lead to cessation of their activity. Environmental studies in southern and northern Europe illustrate this, showing the highest number of species in the summer months. It is during these peaks of the season that the autochthonous transmission of the virus takes place and the occurrence of diseases caused by mosquitoes is observed. The autochthonous transmission of viruses in mosquitoes

in Europe is dominated by the emergence of the number of exotic viruses. The best example of this is the re-occurrence of WNV, which is mainly transmitted by the mosquito species of the genus Culex. The epidemiology of the WNV is complex, and it consists of a cycle of infection between mosquito species that serves as a vector of the virus, wild birds acting as a reservoir and illness in humans and horses. The whole cycle must include populations of mosquitoes that bite birds and mammals. Examples of these populations include the Culex pipiens population (Vogels et al., 2016), especially those in southern Europe, as well as Culex modestus (Balenghien et al, 2008). Models that predict the time and location of human infection with Arboviruses have the potential to more effectively control mosquitoes and prevent disease. The objectives of this study were to analyze and present the occurrence and trend of the West Nile fever in the period 2014-2018, to analyze cases according to the place of residence and determine whether there was a higher incidence of the disease in the flooded area in 2014 as compared with the other parts of the Republic of Srpska, and to present a WNV case classification of patients based on the diagnostic method and the case definition criteria.

## MATERIAL AND METHODS

Using the descriptive method, the demographic data of the patients were analyzed, and the classification of cases based on case definition were performed. The most common clinical forms of the disease and the incidence of the West Nile fever in the period 2014-2018 in the Republic of Srpska were described. All cases reported in the Republic of Srpska were mapped and we analyzed the correlation of the location of the case with the area affected by the floods. Also, each case has been monitored in terms of clinical course of the disease and the outcome of the treatment. Each case has been reported in accordance with the adopted case definition for West Nile fever of the European Center for Disease Control, which is obligatory to be applied in accordance with the current Regulations on the manner of reporting, the contents of records and the content of the notification of infectious diseases (Official Gazette of the Republic of Srpska 07/19). The case can be classified as possible - probable - confirmed on the basis of the case definition criteria.

- Clinical criteria: any person with a high fever or at least one of the following two criteria: encephalitis, meningitis.
- Laboratory criteria: A laboratory test for case confirmation implies at least one of the following four criteria:

- Isolation of WNV from blood or liquor,

- Detection of WNV nucleic acid in the blood or liquor,
- Detection of IgM antibodies specific to WNV in the liquor
- High titre of IgM antibody on WNV and detection of IgG antibody on WNV, and confirmation by neutralization

A laboratory test for a probable case is the antigenic specific response to WNV in serum.

- Epidemiological criteria: at least one of the following two epidemiological links:
- Transfer from animal to human (permanent or shorter stay or exposure to mosquitoes in areas where WNV is endemic in horses or birds),
- Transfer from person to person (vertical transmission, blood transfusion, transplants).

After clinical and epidemiological criteria were confirmed, a sample of blood was taken from each patient and tested for the presence of IgM and IgG antibodies using ELISA according to the manufacturer's instructions (Euroimmun, Germany) at the Institute of Clinical Microbiology at the University Clinical Center of Republika Srpska (UCC RS). Each patient has been monitored in terms of clinical course and outcome.

## RESULTS

In the period 2014-2018, a total of 18 cases of West Nile fever were reported. The incidence ranged from 0.79 in 2014 to 0.43 in 2018, which clearly corresponds with large floods that affected the Republika Srpska in 2014 (Figure 1). Based on a descriptive analysis, the data showed that the average age of patients in the observed period was 51.7 years. The youngest patient was 6 and the oldest one was 87 years old, but there was no statistically significant difference in the number of patients between different age groups. The results of the $\chi \wedge 2$ test ($\chi \wedge 2 = 3.556$; $p = 0.096$) showed that there was no statistically significant difference ($p < 0.05$) in the number between male and female patients. Based on the statistical analysis, it was concluded that there was a statistically significant difference in the number of patients by the type of settlement in which they lived. Statistically significant ($p = 0.018 < 0.05$) was the higher number of patients living in the rural area as compared to the number of patients living in the urban area, exactly 76% of them.

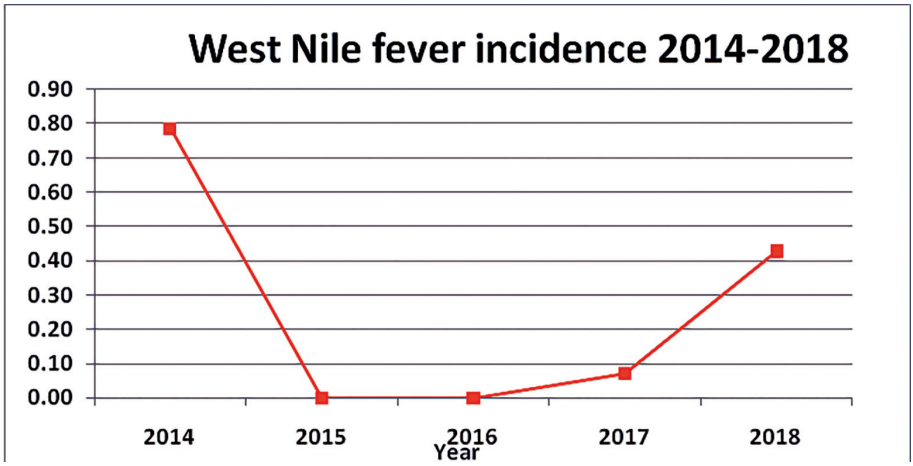


Figure 1. Incidence of the WNF 2014 - 2018 in the Republic of Srpska

The results of χ ^ 2 test (χ ^ 2 = 14,222; p = 0,000) revealed a statistically significant difference in the number of patients according to whether they lived in a settlement that was flooded or not. Significantly higher number of patients lived in settlements that were flooded as compared to the number of patients living in settlements that were not affected by the floods (Figure 2).

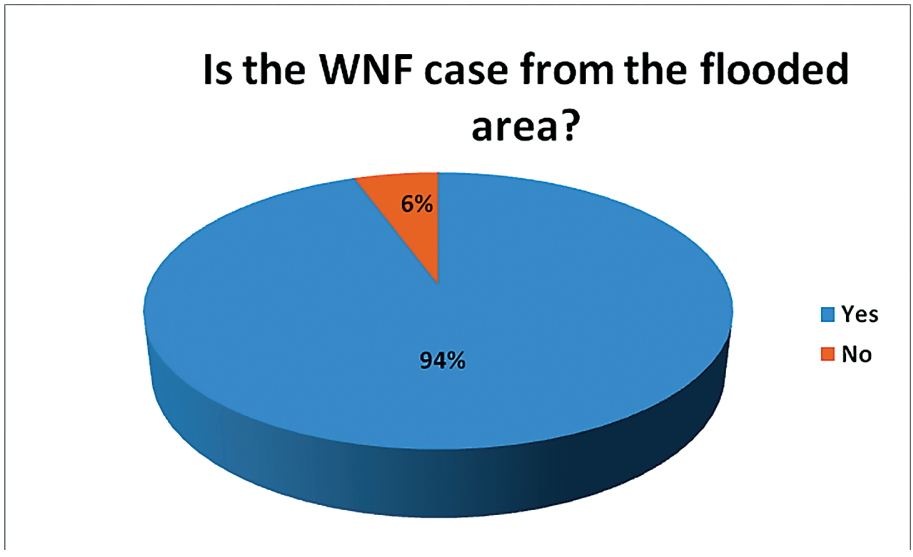


Figure 2. Representation of the West Nile fever cases in the flooded area in the Republic of Srpska

Of the total number of patients, 94% were from the flooded areas belonging to the municipalities of Šamac, Derventa, Modriča, Prijedor, Novi Grad, Banja Luka, Teslić, Petrovo, which corresponds exactly to the map of the areas that were affected by catastrophic floods in 2014 (Figure 3). Out of all cases, 90.9% were reported in June and July 2014, that is, less than a month or two after the flood. The most common clinical form of the disease was neuroinvasive infection with a fever and meningoencephalitis (100%), which fully meets the criteria of the case definition of this disease. All reported cases have been tested at the Department of Microbiology (UCC RS) with a positive finding of serum IgM and IgG antibodies and are reported as probable cases in accordance with the international case definition of this infectious disease. All patients were dissmised completely recovered.

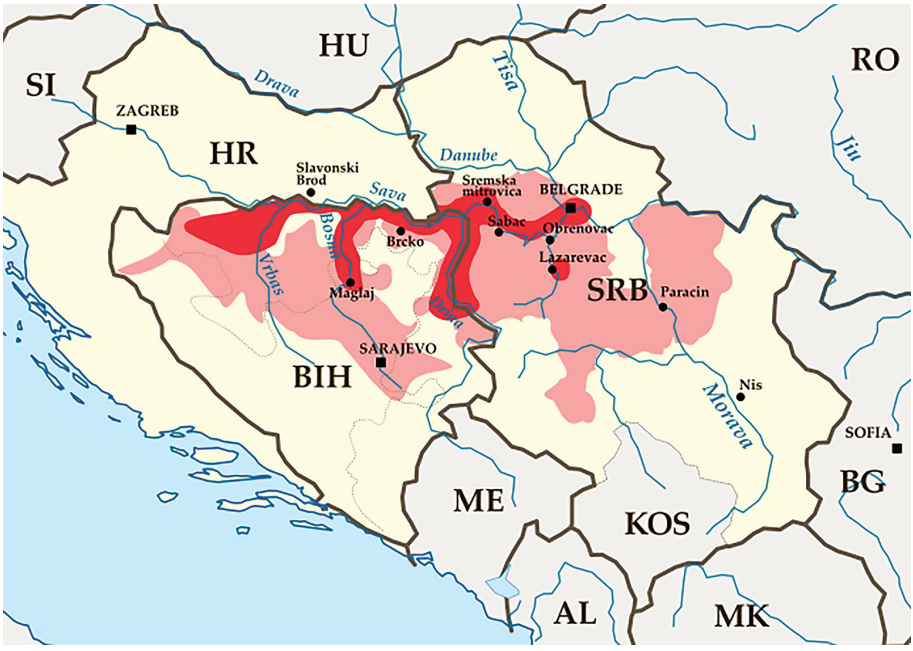


Figure 3. Map of the flooded region in 2014 (Source: Arnold Platon based on European Comission press releases for 16 May and 20 May, ECHO/AFP map, The Guardian map and Al-Jazeera Balkans interactive map,

## DISCUSSION

Since its discovery in 1937, WNV has spread beyond its original geographical area and has caused disease on all continents except in Antarctica. The

main goal of the West Nile fever control in the human population is to continuously monitor the epidemiological, clinical and virological characteristics of the WNV in order to take appropriate measures to prevent and control the spread of the disease. Reported cases of illness and/or deaths from the West Nile fever neuroinvasive form in the human population are the most accurate indicators of the activity of the WNV in humans. It is necessary to evaluate potential risk factors for infection and possible transmission within a period of three weeks before the onset of symptoms of the disease. The case of West Nile fever should be suspected in people over 50 years of age presenting with encephalitis or meningitis in the summer or early autumn months. Restricting the blood donation should be considered in areas where this virus is circulating. There are specific rules in the EU that address the safety of blood by delaying voluntary donations of 28 days after leaving the area in which there is a transmission (EU Commission Directive 2004). The epidemiological situation of the disease in Europe is heterogeneous: some countries report epidemics in humans and animals every year, while others have never reported any autochthonous cases. The concept of "One Health" recognizes that the health of people, animals and the environment is interconnected and that only a collaborative interdisciplinary approach can effectively achieve optimal health outcomes (Lerner et al., 2015).

After the catastrophic floods that affected the Republic of Srpska in 2014, on May 26, 2014, a Webex conference was held with the representatives of Bosnia and Herzegovina, Croatia and Serbia, the World Health Organization and the European Center for Disease Control. The aim of the meeting was to discuss on necessary measures to prevent the outbreak of diseases that are transmitted to the vectors. The main risk identified as a threat to human health is the epidemic of the WNV.

The first case of West Nile fever has been reported to the Institute of Public Health of the Republic of Srpska on 10 June, 2014. This first-reported case was from the Banja Luka area, followed by cases from the municipalities of Novi Grad, Teslić, Derventa, etc. All patients were hospitalized on the basis of relevant clinical symptoms and some were later transferred to further diagnostics and treatment to the University Clinical Center of the Republic of Srpska in Banja Luka. Since all sera of the patients were tested positive for IgM and IgG antibodies using the ELISA, they were all classified as a probable cases. However, University Clinical Center of the Republic of Srpska doesn't sample cerebrospinal fluid for confirmation of the cases, which is a weak spot in the management of the cases and surveillance of the disease. Therefore, this procedure should be implemented in the future according to the European Center

for Disease Control case definition for confirmation (Hrnjakovic Cvjetkovic et al., 2018). This is very important for distinguishing WNV from tick-borne encephalitis and other arboviral infections as they have very similar clinical manifestations (Petrovic et al., 2018). It is important to emphasize that this is a passive surveillance and there were no intensive measures to detect cases using different procedures than usually. The increase of incidence was not the consequence of active surveillance and tracing of the cases. One of the factors that might have influenced such condition was the fact that the population from the flooded area spent more time outside, repairing the damage after the floods and working around artificial water ponds.

That same year 2014, Italy reported the epidemic of West Nile fever in human and equine population caused by the WNV of lineage 1 (Delbue et al., 2014). WNV lineage 2 is responsible for the outbreak of the epidemic in the same year in Hungary and Greece (Hernandez-Triana et al., 2014). This trend of outbreaks continued in the summer and early autumn months throughout the region. In Austria, 2 cases were registered (Kolodziejek et al., 2014) and the reporting continued in 2015 with 8 new cases. In 2018, 2083 cases were reported in Europe. EU member states reported 1503 cases, which is more than 7-fold increase compared to 2017, mostly in Bulgaria, France and Italy. The neighboring countries of the European Union reported 580 human cases, out of which 415 cases were registered in Serbia (ECDC, 2018).

Monitoring of the disease in humans focuses on early detection of cases and identification of affected areas aimed at the implementation of relevant measures including safety standards for blood transfusion, vector control and communication with relevant institutions and the public. This is particularly challenging because most cases are asymptomatic, which is a particular risk in aspects of blood and organs donations.

The integration of surveillance and surveillance activities carried out by the public health authorities, animal health care institutions and institutions responsible for vector control and surveillace should enhance efficiency and save resources by targeted measures (Gossner et al., 2017).

## CONCLUSION

In the period 2014-2018, the highest incidence of West Nile fever was recorded in 2014 when the Republic of Srpska was affected by the floods. All hospitalized and analysed patients had neuroinvasive disease. Most cases were registered in flooded areas mainly among rural populations. It is necessary to perform more analyses in close cooperation with the veterinary sector to

establish and confirm the interconnection between disease cases, floods and infected mosquitoes in the flooded area. All cases are classified as "probable cases"; however, it is crucial to implement internationally endorsed procedures as a clinical alghoritm for the confirmation of the case in accordance with the laboratory criteria for WNV case definition.

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# SEROPREVALENCE OF INFECTION WITH TOXOPLASMA GONDII AMONG THE RESIDENTS OF SOUTH BACKA DISTRICT, SERBIA

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## Abstract

*Toxoplasma gondii* is a ubiquitous coccidian protozoan that can infect a wide range of warm-blooded animals, including humans. For immunocompetent humans it is a well-adapted parasite that usually causes asymptomatic infection. However, in congenitally infected infants and immunocompromised patients it can cause a serious life threatening disease. The aim of this study was to determine the levels of anti-toxoplasma antibodies among residents of the South Backa District, Serbia. During the period from January 2014 to December 2018, sera from 11,288 persons from South Backa District were tested on the presence of IgM and IgG antibodies against *Toxoplasma gondii*, using ELISA test (Euroimmun, Lübeck, Germany). The testing was performed on the automatic device Euroimmun Analyzer I-2P. The avidity of IgG WNV antibodies was determined for IgG positive sera using commercial avidity test (Euroimmun, Lübeck, Germany). In total, out of 11,288 patients who were tested for toxoplasma antibodies, the results were positive for 2,513 (22.26%). In 2014 seropositivity for *Toxoplasma gondii* was 25.78% (464/1800), in 2015 it was 23.30% (400/1717), in 2016 it amounted 20.99% (474/2258), in 2017 it was 21.47% (529/2464) and in 2018 seropositivity was 20.96% (639/3049). Seropositivity of 26.53% (390/1470) was found in males and it amounted 21.62% (2123/9818) in

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females. Possible recent infection within the last 12 months was found in 1.70% (192/11288) patients. Possible acute infection or false - positive IgM result was detected in 1.31% (148/11288) patients. Past infection was found in 2173/11288 (19.25%) patients. Equivocal results were found in 1.48% of samples (167/11288). The lowest frequency of anti-toxoplasma antibodies was detected in pre-school children 13.07% (97/742) and the highest in persons older than 65 years 60.18% (65/108). Comparing the results of the research done from 2014 to 2018 with the data from 1989, a significant decline of seroprevalence in general population and women of generative age was found.

**Key words**: *Toxoplasma gondii*, IgG ELISA, IgM ELISA

# SEROPREVALENCA INFEKCIJE PROTOZOOM TOXOPLASMA GONDII U PRIPADNIKA POPULACIJE JUŽNO BAČKOG OKRUGA

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## Kratak sadržaj

*Toxoplasma gondii* je široko rasprostranjena protozoa sposobna da inficira brojne toplokrvne životinje uključujući i ljude. Životni ciklus je kompleksan sa felidama kao definitivnim i toplokrvnim životinjama kao prelaznim domaćinima. Za imunokompetentne ljude to je prijateljski, dobro adaptiran parazit koji često izaziva asimptomatsku infekciju. Toksoplazmoza može biti teško, po život opasno oboljenje kod kongenitalno inficiranog ploda i imunodeficitarnih osoba. Kako je pokazano u različitim studijama učestalost infekcije toksoplazmom je različita. Cilj ove studije je bio da se odredi učestalost anti-toksoplazma antitela u stanovnika Južno

Bačkog okruga, Srbija. U periodu od januara 2014.god do decembra 2018. god, serumi uzorkovani od 11288 osoba iz Južno Bačkog okruga su testirani na IgM i IgG antitela protiv *Toxoplasmae gondii*, ELISA testom (Euroimmun, Lübeck, Germany). Testiranje je sprovedeno na automatizovanom procesoru Euroimmun Analyzer I-2P. Aviditet IgG antitela za IgG pozitivne serume je testiran komercijalnim testom (Euroimmun, Lübeck, Germany). Od ukupno 11288 osoba koje su testirane na anti-toksoplazma antitela 2513 (22,26%) su bile pozitivne. U 2014. godini u 25,78% (464/1800) uzoraka seruma dokazana su antitela na toksoplazmu, a u 2015. godini seropozitivnost je iznosila 23,30% (400/1717), u 2016. godini 20,99% (474/2258), u 2017. godini 21,47% (529/2464) i u 2018. godini je bila 20,96% (639/3049). Kod muškaraca je utvrđeno 26,53% (390/1470) seropozitivnih dok je kod žena bilo 21,62% (2123/9818) seropozitivnih. Moguća nedavna infekcija, tokom poslednjih 12 meseci je dokazana u 1,70% (192/11288) osoba. Moguća akutna infekcija ili lažno pozitivan IgM rezultat je utvrđen kod 1,31% (148/11288) osoba. Prošla infekcija je dokazana kod 2173/11288 (19,25%) osoba. Granični rezultat je nađen u 1,48% (167/11288) slučajeva. Najniža prevalenca anti-toksoplazma antitela je dokazana u predškolske dece 13,07% (97/742) a najviša u osoba starijih od 65 godina 60,18% (65/108). Poređenjem seroprevalence dobijene studijom iz 2018. sa rezultatima studije iz 1989. godine utvrđena je signifikantno niža prevalenca u studiji iz 2018. godine.

**Ključne reči:** *Toxoplasma gondii*, IgG ELISA, IgM ELISA

## INTRODUCTION

*Toxoplasma gondii* is a ubiquitous coccidian protozoan that can infect a wide range of warm-blooded animals, including humans. The life cycle of this obligate intracellular parasite is complex. Domestic and wild members of the family Felidae are definitive host where it multiplies in the small intestine (sexual cycle). Humans and warm-blooded animals, including most livestock are intermediate hosts, where it forms a cyst in muscular and neural tissues. *T. gondii* causes zoonotic, cosmopolitan disease *Toxoplasmosis.* For immunocompetent humans it is a well- adapted parasite that causes asymptomatic infection in 10 - 20% cases according to studies conducted in USA and Europe. However, in congenitally infected infants and immunocompromised patients it can cause a serious life threatening disease.

## MATERIAL AND METHODS

The prevalence of anti-toxoplasma antibodies was determined retrospectively in 11,288 persons from South Bačka District, during a 5-year period (from January 2014 to December 2018). The analysed group included 9,818 females (aged 1 to 85) and 1470 males (aged 0 to 88). Among females, 9,113 (92.82%) were childbearing women (defined in this paper as 19–45 year-olds). The serum samples were analysed for toxoplasma specific immunoglobulin M (IgM) and G (IgG) antibodies, by ELISA IgG and ELISA IgM test (Euroimmun, Lübeck, Germany). The testing was performed on the automated device Euroimmun Analyzer I-2P at the Institute of Public Health of Vojvodina, Center of Virology. For determination of IgG avidity commercial ELISA using urea as a denaturing factor was carried out according to manufacturer's instruction (Euroimmun, Lübeck, Germany). A relative avidity index (RAI) was calculated and expressed as a percentile dividing the OD values with and without urea treatment.

## RESULTS

During a 5-year period toxoplasma specific antibodies were found in 2,513 (22.26%) out of 11,288 sera. Our results indicate that 76.26% (8608/11288) of tested persons were seronegative. In 1.48% (167/11288) of tested persons, equivocal results of serologic tests were found. In 2014 detected seropositivity for *Toxoplasma gondii* was 25.78% (464/1800), in 2015 it was 23.30% (400/1717), in 2016 was 20.99% (474/2258), and in 2017 seropositivity was detected in 21.47% (529/2464) and in 2018 in 20.96% (639/3049) of tested persons. Seropositivity in 2014 was significantly higher than in 2016 ($p=0.0004$), in 2017 ($p=010011$) and 2018 ($p=0.0001$). Seropositivity of 26.53% (390/1470) was found in males while it was 21.62% (2123/9818) in females. The difference among male and female was statistically significant ($p<0.0001$).

Sole IgM positivity was found in 1.31% (148/11288), sole IgG positivity in 19.25% (2173/11288) and both IgM and IgG in 1.70% (192/11288) of tested population of South Backa District (Table.1). Serologic profile of positive IgM anti-toxoplasma antibodies without IgG antibodies point to possible acute infection or false positive IgM result.

Table 1. The presence of IgM, and IgG antibodies in sera of investigated persons, from 2014 to 2018

| Year | N | IgM positive IgG negative | | IgM positive IgG positive | | IgM negative IgG positive | | Total positive |
|---|---|---|---|---|---|---|---|---|
| | | n | % | n | % | n | % | n |
| 2014 | 1800 | 42 | 2.33 | 46 | 2.55 | 376 | 20.89 | 464 |
| 2015 | 1717 | 13 | 0.75 | 40 | 2.33 | 347 | 20.21 | 400 |
| 2016 | 2258 | 31 | 1.37 | 37 | 1.64 | 406 | 17.98 | 474 |
| 2017 | 2464 | 38 | 1.54 | 48 | 1.95 | 443 | 17.98 | 529 |
| 2018 | 3049 | 24 | 0.78 | 21 | 0.19 | 601 | 19.71 | 646 |
| Total | 11288 | 148 | 1.31 | 192 | 1.70 | 2173 | 19.25 | 2513 |

Legend: N total number of samples; n number of positive samples; IgM immunoglobulin M; IgG immunoglobulin G;

Possible recent infection within the last 12 months was found in 1.70% (192/11288) patients in which both IgG and IgM antibodies were detected. In 2,173 (19.25%) patients a serologic profile consistent with a past infection was found (only anti-toxoplasma IgG antibodies were positive). The results of *Toxoplasma gondii* seroprevalence in population of South Backa District by age group are shown in Table 2.

In the youngest group (0-3 years) seroprevalence varied from 9% (in 2015) to 18% (in 2016). In the eldest group seroprevalence varied from 47% (in 2016) to 68% (in 2014). We found that the seroprevalence for *Toxoplasma gondii* significantly increased with age.

In the population of South Backa District seroprevalence in childbearing women was 20.04% (1826/9113). Out of 9,113 childbearing women 101 (1.1%) had serological profile consistent with possible recent infection within the last 12 months. In 20 childbearing women avidity of IgG was determined and RIA values were in the range from 61.2% to 97% indicating past toxoplasma infection in all tested childbearing women.

Table 2. *Toxoplasma gondii* seroprevalence in population of South Backa District by age group

| Year / Age group | 2014 | 2015 | 2016 | 2017 | 2018 |
|---|---|---|---|---|---|
| 0-3 | 15% (13/86) | 9% (9/98) | 18% (18/101) | 12% (11/88) | 17% (26/150) |
| 4-6 | 12% (6/48) | 11% (6/54) | 9% (4/45) | 0% (0/43) | 14% (4/29) |
| 7-18 | 27% (48/179) | 18% (36/198) | 22% (42/192) | 22% (33/148) | 19% (31/159) |
| 19-25 | 26% (56/212) | 26% (47/177) | 22% (47/213) | 21% (59/284) | 25% (93/377) |
| 26-35 | 22% (180/821) | 19% (144/749) | 18% (199/1093) | 20% (231/1160) | 18% (258/1451) |
| 36-45 | 29% (101/348) | 29% (96/336) | 22% (113/513) | 22% (146/648) | 22% (164/742) |
| 46-55 | 44% (18/41) | 52% (24/46) | 58% (28/48) | 42% (16/38) | 39% (22/57) |
| 56-65 | 64% (29/45) | 63% (26/41) | 44% (15/34) | 59% (19/32) | 42% (21/50) |
| > 66 | 68% (13/19) | 65% (11/17) | 47% (8/17) | 59% (13/22) | 61% (20/33) |

## DISCUSSION

Compared to other countries, a moderate seroprevalence (22.26%) for anti-Toxoplasma specific antibodies was found in our study in South Backa District. The highest seroprevalences of toxoplasmosis were detected in tropical areas in Africa (14%-78%), Brazil (75%) and Ethiopia (73%). The lowest seroprevalences of toxoplasmosis were observed in hot and dry area in Asia (Foroutan-Rad M, 2016). In Chinese blood donors the overall IgG seroprevalence of *T. gondii* infection was low 6.26% (Wang et al., 2018). Seroprevalence of 24.2% (123/508), similar to that detected in our study, was found in a healthy population of Slovakia (Studeničová et al., 2006). In a study of Coroiu and co-workers (2009), the seroprevalence for *T. gondii* in general population of north-western and central Romania were higher, amounting 59.4% (686/1155). In serological study conducted in Germany seroprevalence of 55% (3602/6564) was found (Wilking et al., 2016). The results of many studies of *Toxoplasma gondii* infection in humans showed different rates of seroprevalence depending on geographical location, environment, socio-economic, culinary habits and applied diagnostic methods.

In the study conducted in 1989, we tested 1000 serum samples of person from South Backa District applying immunofluorescent test for anti-toxoplas-

ma IgG and IgM antibodies (Hrnjakovic Cvjetkovic, 1989). Comparing the data of these two studies we found that seroprevalence was decreasing from 30.70% in 1989 to 22.26 % in 2018 ($p<0.0001$). Generally, a decrease in the number of seropositive humans and animals had been detected in many European countries and USA over the last few decades. In the study comparing the U.S. prevalence of *T. gondii* infection in humans during period 1988-1994 and 1999-2004, a decrease from 14.1% to 9.0% in 6 to 49 year-olds was found (Jones et al., 2007). The *T. gondii* overall seroprevalence decreased from 47% in 1979/1980 to 22% (95% CI 20% to 24%) in the Portuguese population in 2013 (Gargaté et al, 2016). In our study seroprevalence increased with age from 15% in the age group of 0 to 3 year-olds to 60% in the age group > 65 years like in other similar studies (Wilking et al., 2016; Studeničová et al., 2006; Gargaté et al., 2016).

Seroprevalence in childbearing women found in this study was 20.04% (1826/9113) and it differs from older data from our country. Among women of reproductive age, in the area of Belgrade, during the 1988-1991 period, the rate of infection with *Toxoplasma gondii* was 77.4% (Bobić et al., 1998). In women of childbearing age in the period from 1990 to 2000 seroprevalence were 11% in Norway, 14% in Sweden and 8% in East England. (Tenter et al., 2000; Petersson et al., 2000)

Comparing seroprevalence in childbearing women in 2014-2018 (20.04%) with seroprevalence in 1989 (33.48%) (Hrnjakovic Cvjetković, 1989), we found that seroprevalence in South Backa District significantly decreased over time. The similar trend was noticed in the studies on seroprevalence of childbearing women from Portugal (Gargaté et al., 2016).

Possible recent infection within the last 12 months of 1.1% was determined in this study of 9,113 women of childbearing age. In the study of Mumcuoglu et al. (2014) 0.9% out of 4,758 pregnant women had the serological profile consistent with possible recent infection. In the study of Stojanovic (1998), acute toxoplasma infection was registered in 0.61% of 1,266 pregnant women in Timok region in 1998.

Due to acquired data on seroprevalence, out of 22.26% in this study, South Backa District is listed among regions of moderate seroprevalence in respect of toxoplasmosis. In the time period from 2016 to 2018, seroprevalence of toxoplasmosis declined significantly in comparison to its values in 2014. Comparing the results of the research performed from 2014 to 2018 with the data from 1989, there is a significant decline of seroprevalence in general population and women at generative age, which is in compliance with the studies conducted in the USA and the EU.

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# COMPARATIVE STUDY OF WATER CONTENT IN HONEY PRODUCED IN DIFFERENT YEARS

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## Abstract

One hundred thirty-three honey samples of different floral origin from two different production years (2017 and 2018) from Serbia were examined in order to determine water content. The water content in honey affects physical, microbiological, sensory properties, and commercial value of honey. The water content in all examined honey samples produced in 2017 (56) was below maximum permissible level set by local regulations for honey. Out of the total of 77 tested honey samples that were produced in 2018, 3 samples (3.9%) did not comply with the provisions of the Regulation. By analysing honey samples originating from 2017, there was a significant difference between the water contents betwen linden honey and honeydew ($p$ = 0.0027). The same result was obtained based on the water content in different honey types from the year 2018 ($p$ = 0.00022). Using the F test, it has been shown that there is no significant difference in the water content between certain types of honey produced in these two years ( 2017 and 2018).

**Key words*:*** honey, water

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# KOMPARATIVNA ANALIZA SADRŽAJA VODE U MEDU PROIZVEDNOM U RAZLIČITIM GODINAMA

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## Kratak sadržaj

Ispitano je 133 uzorka meda različitog biljnog porekla iz dve različite proizvodne godine (2017. i 2018.), poreklom iz Srbije, kako bi se odredio sadržaj vode. Sadržaj vode u medu utiče na fizičke, mikrobiološke, senzorne osobine i komercijalnu vrednost meda. Sadržaj vode u svim ispitivanim uzorcima meda proizvedenim u 2017. godini (56) bio je ispod maksimalno dozvoljenog nivoa utvrđenog Pravilnikom o kvalitetu meda. Od ukupno 77 testiranih uzoraka meda koji su proizvedeni u 2018. godini, u 3 uzorka (3,9%), sadržaj vode nije odgovarao uslovima Pravilnika. Analizom uzoraka meda iz 2017. godine utvrđena je statistički značajna razlika u sadržaju vode između lipovog meda i medljikovca ($p = 0,0027$). Statistička značajnost utvrđena je i za proizvodnu 2018. godinu ($p = 0,00022$). Primenom F testa, nisu utvrđene značajna razlika u sadržaju vode između pojedinih vrsta meda iz dve proizvodne godine (2017. i 2018.).

**Ključne reči:** med, voda

## INTRODUCTION

Natural honey is sticky, viscous solution containing about 80–85% carbohydrates (mainly glucose and fructose), 15–17% water, 0.1–0.4% proteins, 0.2% ash and minor amounts of amino acids, enzymes, vitamins and other substances such as phenolic antioxidants (Buba et al., 2013; Kek et al., 2017). Honey is a natural food consumed without any processing and is characterized by its complex composition, which varies in accordance with the bee species, geographical region, available floral source and storage conditions (Karabagias et al., 2014). Serbia has a very long tradition of beekeeping. Its favourable climate, good geographical conditions and a variety of botanical species provide a great potential for the development of apiculture (Jarić et al., 2013).

The water content (moisture) in honey depends on the production season, floral source, abundance of nectar flow, soil, ventilation of the beehive, colony

strength, and meteorological conditions in the area of honey production, primarily air humidity (Escuerdo et al., 2014; Kirs et al., 2011; Lazarević et al., 2017; Sousa et al., 2016). An important factor that could affect the water content is honey maturation and harvest time. According to most of national good beekeeping practice recommendations, beekeeper should harvest honey when at least 2/3 wax combs in frames are covered with wax lids.

Moisture substantially affects some physical properties of honey (crystallization, viscosity, rheological behaviour). Other parameters such as appearance, colour, palatability, taste, specific weight, solubility, conservation, and to a large extent commercial value of honey are also important (Azeredo et al., *2003;* Escuerdo et al., 2014; Prica et al., 2014). High moisture of honey is also an indicator of honey adulteration (Nyau et al., 2010; Obiegbuna et al., 2017). The water in honey is of major importance to its stability against fermentation and granulation. The low water content protects honey from microbiological activity and thus it can be preserved for longer periods (Akhtar et al., 2014). The higher the moisture, the higher the probability that honey will ferment upon storage by osmotolerant yeasts (Viuda-Martos et al., 2010). Tosi et al. (2004) suggested that inhibition of honey fermentation occurred when water content is below 17.1 %. Besides, honey stability is affected by the microbiological load for moisture content between 17.1% and 20%, while osmophilic yeasts may develop when the moisture contents are higher than 20%. Moreover, according to Snowdon and Cliver (1996) honey with water content over 17% is susceptible to fermentation and honey with more than 19% of water is very like to ferment. However, Boateng and Diunase (2015) stated that the levels of water between 13 and 25% are low and that yeast fermentation and bacterial growth will not be allowed. Furthermore, glucose/water ratio (G/W) was also recognised as useful for predicting the crystallisation of honey (Manikis and Thrasivoulou, 2001) and consequently the possible increase in number of microorganisms.

In accordance with the regulation concerning the quality of honey in the Republic of Serbia (Official Gazette, 101/2015), maximum value of water content in all types of honey (except in baker's honey) is set on 20%.

The purpose of this study was to determine water content in honey collected during 2017 and 2018, in order to obtain the information about the honey quality and safety. In addition, analysis of variance and F test were applied to determine the significance of statistical differences between the water contents in different honey types and in production years.

## MATERIAL AND METHODS

### Honey samples

A total of 133 honey samples were collected directly from beekeepers. The collected samples were produced in different regions of Serbia. In the year 2017 the total of 56 samples were collected, while the number of samples from 2018 was 77. All collected samples were in their original packaging and transferred to the laboratory of Scientific Veterinary Institute "Novi Sad" for examination. Honey analyses were carried out immediately after sampling.

A total of 133 examined samples included 55 samples of meadow honey, 35 samples of acacia honey, 13 samples of linden honey, 12 samples of honeydew, 11 samples of polyfloral honey and 7 samples of sunflower honey.

### Water content analysis

Water content was determined by refractometry, measuring the refractive index (RI) according to Harmonised methods of the International Honey Commission Methods (2009), using a standard model Abbetype refractometer a 20° C. Water content (%) was then obtained from the Chataway table.

### Statistical analysis

Statistical analysis was performed by the PAST software package, version 2.12, Oslo, Norway. Data were grouped according to the type of honey and presented as mean ± standard error, minimum and maximum values. Statistical data analysis included analysis of variance (one-way ANOVA), as well as Tukey's pairwise comparison. Using the F test, it was examined whether there is a significant difference between the water content in certain types of honey in those two years.

## RESULTS AND DISCUSSION

Average values of water content in different honey types produced in 2017 and 2018, obtained in this study are summarized in Table 1. The obtained values were compared with the values that are prescribed by Regulation on the quality of honey in the Republic of Serbia (Official Gazette, 101/2015). The results were compared with the results from other authors from Serbia and other countries. The water content in all investigated honey samples produced

in 2017 was below 20%, which is the maximum permissible level set by local regulations for honeys (Official Gazette, 101/2015). Our results also demonstrated a low water content of honeydew honey and high water content of linden honey, compared to other examined honey types. Out of the total of 77 tested honey samples that were produced in the 2018, 3 samples (3.9%) did not comply with the provisions of the Regulation.

Table 1. Water content of different types of honey in three production years

| TYPE OF HONEY | Total no. of samples | Water content (%) | | | |
|---|---|---|---|---|---|
| | | Production year | | | |
| | | 2018 | | 2017 | |
| | | No. of samples | $\bar{x}$ ± SD<br>Range | No. of samples | $\bar{x}$ ± SD<br>Range |
| Meadow | 55 | 37 | 16.5 ± 1.2<br>14.0 - 18.0 | 18 | 17.3 ± 1.0 $^{bxy}$<br>15.2 - 19.0 |
| Acacia | 35 | 19 | 16.2 ± 1.4<br>13.8 - 20.8 | 16 | 17.0 ± 1.0$^{bxy}$<br>15.8 - 19.0 |
| Linden | 13 | 7 | 18.9 ± 1.8$^{ax}$<br>15.4 - 20.6 | 6 | 18.2 ± 0.9 $^{ax}$<br>17.2 - 19.4 |
| Polyfloral | 11 | 2 | 17.1 ± 0.1<br>17.0 - 17.2 | 9 | 17.2 ± 1.2<br>15.6 - 19.6 |
| Honey-dew | 12 | 6 | 15.2 ± 0.8$^{by}$<br>14.4 - 16.4 | 6 | 16.2 ± 1.4 $^{by}$<br>14.2 - 18.2 |
| Sunflower | 7 | 6 | 17.4 ± 1.1<br>16.0 - 18.6 | 1 | 15.6<br>15.6 |

$^{a,b}$ $p< 0.05$ $^{x,y}$ $p< 0.01$

Within the group of linden honey, water content was above 20% in 2 out of 7 tested samples (28.6%). In the group of acacia, water content was above 20% in 1 out of 19 tested samples (5.3%). Similarly to the results for honey produced in 2017, the data indicated a low water content of honeydew honey and high water content of linden honey, as compared with other examined honey types. In a study conducted on honey produced in 2013, out of the total number of 50 honey samples, the content of water did not comply with the provisions of the Regulation in only one sample (Prica et al., 2014). Water content in the examined samples ranged between 14.2 and 20.2%, with an average of 16.5±1.01%. In this study, we also established a low water content of honeydew, but high water content of polyfloral honey.

By analysing honey samples from the year 2017, there was a significant difference between the water content in linden honey and honeydew ($p = 0.0027$). The same result was obtained for water content in different honey types from the year 2018, meaning that there was a significant difference at the 0.01 level between honeydew and linden honey ($p = 0.00022$). Additionally, for samples from 2018, there was a significant difference between meadow and linden honey ($p = 0.037$), as well as between acacia and linden honey ($p = 0.012$), at the 0.05 level.

The F test showed that there is no significant difference in the water content between certain types of honey produced in the two years. In terms of the average weather conditions, the vegetation period was warmer and drier in 2017, while the vegetation period of 2018 was also warmer, but somewhat humid (RHSS, 2017-2018). However, despite the differences in climate conditions between the two observed years, we did not find statistically significant differences in the content of water in honey produced in 2017 and 2018. The water content of all groups of honey are summarized for both production years (2017 and 2018) and are shown in Figure 1.

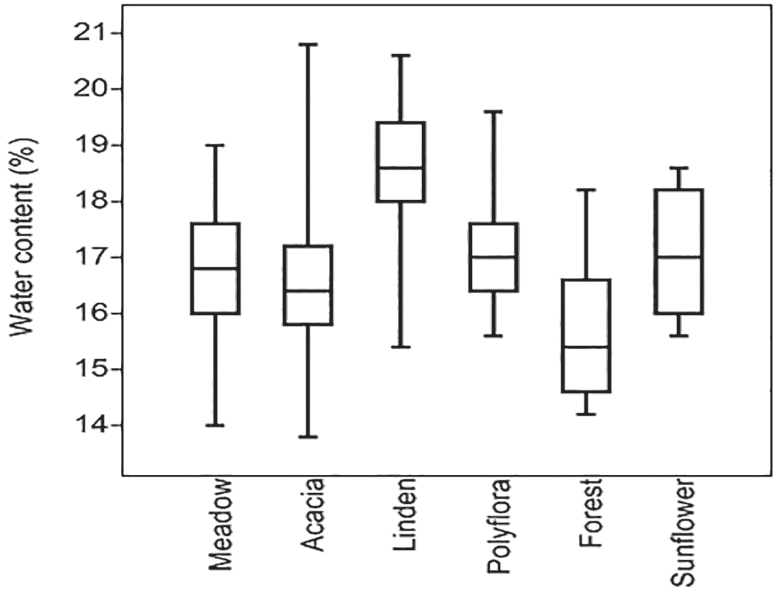


Figure 1. Water content summarized for both production years (2017 and 2018)

Box and whisker plots (Figure 1) compare the water content. The horizontal line in the centre of the box represents the median. The crossed vertical lines above and below the box represent the maximum and minimum values.

Similar values for water in honey are reported by other authors from our and other countries (Vranić et al., 2017; Acquarone et al., 2007; Boussaid et al., 2018; Chakir et al., 2016; Kirs et al., 2011; Sousa et al., 2016; Esccuerdo et al., 2014; Karabagias et al., 2014).

The water content in honey affects physical, microbiological, sensory properties, and commercial value of honey. Honey contains concentrated water solution of two main sugars: fructose and glucose, with small amounts of various complex sugars (Escuredo et al., 2014; Valdés-Silverio et al., 2018). Important aspect of carbohydrate composition in honey is crystallization ability. Over time, liquid honey tends to crystallize. Glucose, the main sugar component of most honeys, may precipitate out spontaneously in the form of glucose monohydrate, and the solution then reverts to the more stable saturated state (Zamora and Chirife, 2006). The moisture substantially affects crystallization process, so it is very important to monitor and control its content in honey. Glucose/water (G/W) ratio is the parameter indicating the ability of honey to crystallize. Generally, honeys with low G/W ratio do not crystallize easily (Escuredo et al., 2014). Mainly, honeys crystallizes faster with G/W ratio higher than 2.16%. On the other hand, honeys with G/W ratio lower than 1.70% remain liquid for a longer period of time (Smanalieva and Senge, 2009). It should be considered that G/W ratio is not always an appropriate index for honey crystallization tendency, so it should be taken only tentatively (Manikis and Thrasyvoulou, 2001; Pascual-Maté et al., 2018). Crystallization of honey is an undesirable process because it affects honey processing during extraction, filtration, mixing and bottling (Dobre et al., 2012; Laos et al., 2011). Crystalized honey is less appealing to the consumer, who prefers it liquid and transparent (Kabbani et al., 2011). *T*osi et al. (2004) noted that crystalline and liquid phases may coexists one period of a time during honey crystallisation and in such liquid phase water activity increases due to the fact that formation of crystal leads to the release of water from the solid phase. Finally, the normally present microorganisms in honey develop and consequently lead to sensory modification of honey and alteration in quality. Yeasts and spore-forming bacteria are commonly found in honey (Finola et al., 2007). According to the results reported by Zamora et al. (2006) the concentrations of the yeasts are in correlation with the water availability. Furthermore, total plate counts, coliforms and yeasts can be used as indicators of the sanitary quality of honey (Naman et al., 2005). The knowledge about the moisture content affecting growth

of microorganisms in honey is important for control of the spoilage of honey (Snowdon and Cliver, 1996). The type of honey and moisture content are important factors affecting a number of microorganisms in honey (Namini et al., 2018). The growth of microorganisms might be controlled by the intrinsic properties of honey such as pH, water content, oxidation-reduction potential, nutrient content, etc. (Iurlina and Fritz, 2005).

## CONCLUSIONS

The study indicated that water content in the examined honeys varied according to botanical origin. The content of water in all examined honey samples produced in 2017 (56) was below the maximum permissible level. Out of the total of 77 tested honey samples produced in 2018, the water content did not correspond to the requirements of the Regulation in 3 samples (3.9%).

Statistically significant differences for water content have been established between linden honey and honeydew. There was no significant difference in the water content between certain types of honey produced in the two years (2017 and 2018). The results obtained by applying routine analysis of water content may be useful for comparing our research with similar studies from other regions and can contribute to the information relevant for predicting the crystallization, viscosity, rheological behaviour of honey as well as microbiological quality.

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# CURRENTLY UNKNOWN ASPECTS OF POULTRY NECROTIC ENTERITIS PATHOGENESIS

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## Abstract

Necrotic enteritis (NE) or poultry clostridiosis is a disease which poses enormous health problems and makes tremendous economic losses to intensive poultry production worldwide. Despite having been targeted in extensive research for decades, a number of aspects of its pathogenesis remain unknown. For more than 30 years alfa-toxin has been considered to be the main virulence factor of the causative agent, but experimental research using a mutant *Clostridium perfringens* strain lacking the gene coding for this confirmed that alpha-toxin is not necessary for pathogenesis. Since the 1980s, NetB toxin has been the main suspected virulence factor. However, recently it has been discovered that the large clostridial cytotoxin named TpeL also contributes to the pathogenesis of NE. In spite of that, the prevalence of the genes which code for these toxins vary between the isolates of *C. perfringens* from the intestines of diseased poultry, which made clear that further investigation into their roles is necessary. It has been agreed that specific intestinal environmental conditions, which favour the growth and multiplication of *C. perfringens*, are key factors to the emergence of disease. Given that a battery of non-specific factors contributes to pathogenesis, as well as that it is impossible to eliminate them in intensive poultry production, not much hope remains that NE can be controlled. In this short review, the current knowledge on the pathogenesis of NE has been summarized.

**Key words**: *Clostridium perfringens*, necrotic enteritis, poultry, NetB, TpeL

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# I DANAS NEPOZNATI ASPEKTI PATOGENEZE NEKROTIČNOG ENTERITISA ŽIVINE

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## Kratak sadržaj

Nekrotični enteritis (NE) ili klostridioza živine, predstavlja veliki zdravstveni problem i nanosi ogromne ekonomske gubitke intenzivnoj živinarskoj proizvodnji širom sveta. Brojni aspekti patogeneze NE su i danas nepoznati, uprkos tome što su decenijama predmet intenzivnih izučavanja. Više od 30 godina je alfa-toksin razmatran kao glavni faktor virulencije uzročnika, ali su eksperimentalna istraživanja primenom mutantnog soja *Clostridium perfringens*, koji nema gen koji kodira sintezu alfa-toksina, potvrdila da ovaj toksin nije neophodan za nastanak bolesti. Od osamdesetih godina prošlog veka, NetB toksin je "glavni osumnjičeni" faktor virulencije, a od nedavno se smatra da i veliki klostridijalni ekstracelularni citotoksin, koji je nazvan TpeL, doprinosi patogenezi NE. Međutim, prevalencija gena koji kodiraju ove toksine, veoma varira kod izolata *C. perfringens* iz creva obolele živine i jasno je da su neophodna dodatna ispitivanja njihove uloge. Saglasnost postoji da su za pojavu bolesti ključni specifični uslovi u crevima koji pogoduju rastu i umnožavanju *C. perfringens*. Ako uzmemo u obzir da niz nespecifičnih faktora tome doprinosi, kao i da ih je praktično nemoguće u potpunosti otkloniti u intenzivnoj živinarskoj proizvodnji, ne ostaje mnogo nade da je nekrotični enteritis moguće staviti pod kontrolu. U radu ukratko sumiramo trenutna stanovišta o patogenezi NE živine.

**Ključne reči:** *Clostridium perfringens*, nekrotični enteritis, živina, NetB, TpeL

## INTRODUCTION

Poultry clostridiosis, also known as necrotic enteritis (NE), was first reported in Australia (Bennetts, 1930), but it took three decades to be described in detail in the United Kingdom (Parish, 1961). However, it was not listed in a British guide to common poultry diseases in 1964, nor in a review article on

global poultry production and health problems in 1979. What is more, even in 1997, it still failed to attract much attention in an international directory of poultry diseases. It was not until the beginning of the 21st century that NE started to be considered a global issue (Williams, 2005). A possible reason for the emergence of this problem is a continuous administration of antibiotics as growth promotors, for example virginiamycin and bacitracin, as well as anticoccidials, especially those of the ionophorous group. Besides being coccidiostats, the latter is known for their Gram-positive antibacterial activity which inhibits *C. perfringens* growth in the guts and these contributes to its antimicrobial resistance. The use of antibiotics other than those with coccidiostatic and histomonostatic effects added to broiler feed has been forbidden in the EU since 2006 (Regulation 1831/ 2003). A sharp rise in incidence of NE has resulted from the ban on growth-promoting antibiotics and the withdrawal of ionophore coccidiostats owing to vaccination against coccidia (Coper and Songer, 2009). Today NE makes huge losses to intensive poultry production worldwide, which are estimated to may reach about 2 billion US dollars per annum (Martin and Smyth, 2009; Coper and Songer, 2009). The disease mainly occurs in broiler chickens 2-6 weeks after hatching, or more precisely in those 4-weak-old, although cases have been described in older birds, even up to 6 months (Williams, 2005; Paiva and McElroy, 2014). In peracute and acute cases, mortality is high, sometimes reaching 50%, often without premonitory signs (Timbermont et al., 2011). Nowadays, for reasons not clear enough, subclinical diseases prevail (Van Immerseel et al., 2008). The losses result from reduced growth, increased feed conversion and carcass condemnation at slaughter (Bailey et al., 2013). Typical pathology is detected in the jejunum and ileum, which varies from multifocal ulcerations in less severe cases to greenish or yellowish pseudomembranes if extensive mucosal inflammation and necrosis develop (Ficken and Wages, 1997). Enabling bacteria, including *C. perfringens,* to enter the portal blood circulation, the intestinal damage may lead to cholangiohepatitis. Such livers are enlarged, pale, yellowish, with haemorrhages and white necrotic foci. These changes are mainly detected in abbatoirs, without signs in the flock which would suggest the pending outcome.

In spite of extensive research in the last several decades, NE still remains a disease with some aspects not entirely clear, or even unknown. This specially refers to virulence factors of the bacterium, which are of utmost importance to the pathogenesis.

## THE CAUSATIVE AGENT AND SOURCES OF INFECTION

The causative agent of NE is *Clostridium perfringens*, an anaerobic spore-forming bacterium, widely distributed in nature: it can be detected in soil, dust, sewage, and fresh and marine water. Besides, it is a common inhabitant of the intestinal tract in both humans and other homeotherms (Songer, 1996; Brynestad and Granum, 2002). Depending on the production of four mayor toxins (alpha, beta, epsilon and iota) *C. perfringens* strains are classified in one of the five toxin types – from A to E (Hateway, 1990; Petit et al., 1999). These bacteria may produce at least 13 other toxic or potentially toxic exoproteins, although not one of them may produce all (Coper and Songer, 2009; Chen and McClane, 2015). As a rule, the causative agent of NE is of type A and rarely C (Songer et al., 1996).

Poultry continually ingest *C. perfringens* spores. They are found in poultry housing, as are isolated from the walls, floors, fans, from the water pipes, nipple-drinkers and drip-cups even before the chicks enter (Williams et al., 2005). In addition, poultry feed is frequently contaminated with *C. perfringens* spores, either from raw ingredients, or when processed, stored or distributed. For example, in Brazil, out of 80 raw samples of fresh feed ingredients (meat and bone meal, blood and feather meal, poultry viscera meal and vegetable mix samples), 60% were found to be positive for *C. perfringens* (Casagrande et al., 2013). Thus, feed contamination depends on its composition, the hygiene during production and the storage conditions, and is directly proportional to the levels of soil and faecal contamination (Wojdat et al., 2006; Milanov et al., 2018). Thermal treatments, the pellet and extrusion processes, typically involve exposure to about 85°C for only a few minutes, which is not high enough to kill *C. perfringens* spores (Williams, 2005). However, despite the lack of absolutely effective treatment to prevent contamination with *C. perfringens* spores, poultry feed is not necessarily contaminated while being produced. As *C. perfringens* is ubiquitous in farm environment, even if non-contaminated feed is brought in, 2 weeks after the introduction of chicks, spores are detectable in feed samples (Williams, 2005). However, the intake of *C. perfringens* spores will not inevitably lead to disease.

*C. perfringens* occurs naturally in healthy chickens' guts, not unlike some other bacteria, for example *Lactobacillus* and *Streptococcus* (Knarreborg et al., 2002). It is known that *C. perfringens* may invade the chick's intestines soon after hatching (Paiva and McElroy, 2014) and can be isolated from eggshell fragments, chicken fluff, and paper pads in commercial broiler hatcheries (Craven et al., 2001). Moreover, even newly hatched chicks may be carriers due to verti-

cal transmission of the bacteria (Shane et al., 1984). Insufficiently established gut microbiome, which plays an important role in natural defence against enteric pathogens and the immunity as a whole, hugely contributes to the susceptibility of young birds to NE. Intestinal microbiota decrease the susceptibility to pathogenic bacteria by competing for attachment sites, producing volatile fatty acids and thus shifting the pH to lower values. It has been proven that germ-free chickens were more sensitive to *C. perfringens* or its toxin in comparison not only to those with normal microbiota, but also to those invaded exclusively by *Lactobacillus acidophilus* or *Streptococcus faecalis* (Fukata et al., 1991). It is perfectly possible that changes in the guts, for example reduction in major intestinal microflora, lead to higher numbers of *C perfringens*, and consequently higher production of alpha-toxin which eventually results in the outbreak of NE (Fukata et al., 1991).

## PREDISPOSING FACTORS

The presence of *C. perfringens* alone does not necessarily lead to disease development (Paiva and McElroy, 2014): firstly, its growth in the guts is naturally limited, and secondly, not every strain is capable of producing a disease. The limitation of *C. perfringens* growth in the intestines, which is an environment with limited amino acid available, is due to its incapacity to synthesize enzymes necessary for the production of 13 amino acids (Brynestad and Granum, 2002; Cooper and Songer, 2009; Antonissen et al., 2014). Thus, it is considered that the key factor in the emergence of NE is the intestinal environment which favours the growth of *C. perfringens* and the consequential production of extracellular toxins which will do damage to the intestines (Keyburn et al., 2006; Cooper and Songer, 2009). This can be supported by a number of factors: dietary factors, immune status, stress and comorbidity, and mycotoxins.

*Dietary factors*: Diets with high levels of indigestible, water-soluble non-starch polysaccharides (wheat, rye, oats and barley), protein-rich diets containing relatively high concentrations of poorly digestible proteins result in high protein concentrations in the gastrointestinal tract and thus act as substrates for the bacteria (Jia et al., 2009; Williams, 2005). High percentages of animal protein sources (fishmeal, meat and bone meal) (Drew et al., 2004) and animal fat (Knarreborg et al., 2002) in a diet, as well as decreased intestinal motility (Coper and Songer, 2009) can increase the odds of NE development. High protein content contributes to the increase in pH, which favours the growth and reproduction of *C. perfringens* (Williams, 2005). In chickens artificially infected with *C. perfringens* it was proven that the intake of feed contaminated

with deoxynivalenol (DON), a major mycotoxin, in concentrations of 3,000 to 4,000 μg/kg feed led to more than two-fold increase in the proportion of birds with subclinical NE in comparison with the control (Antonissen et al., 2014). The results undoubtedly showed that DON disrupts intestinal barrier and produces intestinal epithelial damage, which leads to an increased permeability of the gut wall and decreased absorption of dietary proteins, whose concentrations were significantly increased in the duodenal content. What is more, DON did not affect the *in vitro* growth, production of alpha-toxin and netB toxin transcription in *C. perfringens*. In other words, it influenced the gut wall architecture, but not the bacterium pathogenic potential. The resulting leakage of plasma proteins into the gut leads to higher protein concentrations in the intestinal lumen, and favours the growth of *C. perfringens*. In was concluded that feed contaminated with DON at concentrations even below the EU maximum acceptable level (5.0 mg/kg of complementary or complete feeding stuffs), is a predisposing factor for NE in broiler chickens (Antonissen et al., 2014).

*Immune status, stress and comorbidity*: Various stressors can also contribute to the development of NE, such as alterations in the feeding regime (replacing starter diets with grower diets, for example), or increase in stocking density. Comorbidity which lead to immunosuppression (simultaneous infection with some viruses: chick anaemia virus, Gumboro disease or Marek's disease viruses) is also detrimental to the integrity of the gut mucosa. The most well-known predisposing factor for NE is mucosal damage caused by various coccidia species, especially *Eimeria maxima*, due to their intracellular multiplication (Baba et al., 1997; Williams, 2005). It was suggested that even infection with roundworms, namely with Ascaridia species, may contribute to NE due to the intestinal wall damage caused by their larval development (Palliyeguru et al., 2014)

The role of predisposing factors is of utmost importance since in unfavourable intestinal conditions, even highly virulent *C. perfringens* strains fail to produce a disease (Coper and Songer, 2009; Paiva and McElroy, 2014). In healthy chicks' gut content the number of *C. perfringens* usually is up to $10^2$ or $10^3$ CFU/g (Baba et al., 1997), whilst in the intestines of birds suffering from NE the corresponding numbers may increase up to $10^6$ or even $10^8$ CFU/g (Cooper and Songer, 2009). However, even extremely high numbers of *C. perfringens* in gut contents do not inevitably cause NE (Coper and Songer, 2009), since not all strains are able to do so – only clostridia which possess host-specific virulence factors are pathogenic for poultry (Timbermont et al., 2011). Now it is known for sure that firstly, only certain strains are capable of inducing NE, secondly, it happens if some predisposing factors are present, and thirdly, these make up only a minority in the guts of healthy chickens (Timbermont et

al., 2011). Pulse-field gel electrophoresis (PFGE) or amplified fragment length polymorphism (AFLP) detected various genotypes of *C. perfringens* type A in healthy flocks, even within an individual and within the same part of intestine. PFGE proved a high degree of genetic diversity in isolates from healthy birds but little in those suffering from clinical NE (Cooper and Songer, 2009; Timbermont et al., 2009). In naturally infected diseased chicks, a single clone of *C. perfringens* is dominant in the intestines (Timbermont et al., 2011). Isolates of *C. perfringens* from a flock with NE generally belong to the same genotype, even if obtained from different animals or organs (Engström et al., 2003; Nauerby et al., 2003; Gholamiandehkordi et al., 2006). By contrast, Johansson et al. (2010) assessed the genetic diversity of 88 *C. perfringens* isolates from a single broiler flock affected by mild NE in Sweden and detected 32 genotypes with PFGE. In the majority of affected birds more than one genotype was identified, which was explained with the presumption that in mild NE not one virulent strain can achieve complete dominance as it is possible in acute diseases.

## CLOSTRIDIUM PERFRINGENS VIRULENCE FACTORS

Despite the fact that NE has been known for such a long time, *C. perfringens* virulence factors which are of profound significance to the pathogenesis are yet to be identified. For more than 20 years alpha (a) toxin, a zinc-dependent phospholipase/sphingomyelinase C, was considered to be the main factor of virulence in the pathogenesis of NE (Songer, 1996; Van Immerseel et al., 2008), although its precise role in disease development has not been understood fully (Keyburn et al., 2006). Al-Sheikhly and Truscott (1977), for example, successfully reproduced NE in birds infused intraduodenally with bacteria-free crude toxin. In this research it was revealed that alpha-toxin is the most dominant protein present in crude supernatants but the others were neglected, although they could possibly add to its effects or even lead to NE. Earlier studies which confirmed the role of alpha-toxin in chicks inoculated with the supernatant of *C. perfringens* cultures have a certain drawback: they did not take into account the possible presence of some other toxins. The most solid evidence that alpha-toxin is not the key virulence factor is the experimentally induced NE with a mutant lacking alpha-toxin (Keyburn et al., 2006). The role of alpha-toxin in the pathogenesis of NE was doubted also in the research conducted by Thompson et al. (2006). All toxotypes of *C. perfringensa* produce alpha-toxin, but only some of them, types A and C, can cause NE in chickens. It is clear that, if alpha-toxin were sufficient to cause the disease, all *C. perfringens* types would be the causative agents of NE, which obviously is not true.

Some further research has also suggested that alpha-toxin plays only a minor role in the pathogenesis of NE disease. NE in chicks is characterised by granulocyte migration to the intestinal lumen (Olkowski et al., 2006), which is a reaction very different from the leukostasis and lack of inflammatory response induced by alpha-toxin in gas gangrene. These facts additionally support the theory that NE lesions are mediated by some other toxins rather than alpha-toxin. *In vivo*, the substrates for alpha-toxin are phosphatidylcholine and sphingomyelin, which are both components of biological membranes, and so of the guts' epithelial cells. However, the tissue lesions in the early phases of NE do not correspond to the activities of phospholipase and sphingomyelinase of the alpha-toxin (Olkowski et al., 2008).

Keyburn et al. (2008) used a gene knockout mutant and discovered a novel pore-forming toxin – NetB - in a *C. perfringens* strain isolated from chickens with NE in Australia, claiming that NetB is a major virulence factor associated with this chicken disease. Regarding the amino acid sequences NetB toxin is partially similar to some other pore-forming toxins, for example to beta toxin of *C. perfringens* (38% identity) and alpha-toxin produced by *Staphylococcus aureus* (31% identity). NetB forms pores of at least 1.6 nm in diameter in cellular membranes, causing an influx of ions (Ca, Na, Cl etc.) which eventually leads to osmotic cell lysis (Savva et al., 2013). Mature NetB toxin is of similar molecular size to mature beta-toxin (33.2 kDa vs 34.8 kDa), but two share limited sequence identity (38%): phylogenetic analysis indicated that NetB is clearly a distinct toxin which does not belong to the beta-toxin clade (Keyburn et al., 2008). NetB was only identified in the majority (14 out of 18) of *C. perfringens* type A isolates from chickens suffering from NE, but not in 32 isolates which comprised those originating from birds not having the disease and from humans, pigs, cattle and sheep (Keyburn et al., 2008). However, in the initial research on NetB, 4 out of 18 strains capable of causing NE proved negative for the presence of netB in a PCR assay and did not produce NetB, which was confirmed in a Western blot test. Thus, it was suggested that in some strains NetB is not an essential component of virulence (Keyburn et al. 2008). By contrast, in Iran, the netB gene was first detected in a small percentage (7.77%) in chickens with NE in organic broiler farms (Ezatkhah et al., 2016**).**

Johansson et al. (2010) used PCR to investigate into the genetic diversity and prevalence of the gene coding for NetB toxin in *Clostridium perfringens* isolated from a broiler flock affected by mild NE in Sweden. Out of the 34 isolates from NE lesions in 18 birds, 31 (91%) were found to be positive for *NetB* gene (Johansson et al., 2010). However, out of the 23 isolates taken from the stomach and caecums of six birds without NE lesions, in 16 (70%) the *NetB*

gene was confirmed. It was concluded that 1) mild NE can be associated with NetB, but not with the specific *C. perfringens* genotype, 2) NetB can easily be transmitted between various genotypes, and 3) that some other virulence factors may decide whether a severe NE disease will develop or not, for instance, it can be the ability of certain strains to inhibit the growth of less pathogenic or apathogenic *C. perfringens* strains and establish dominance and lead to severe NE disease (Johansson et al., 2010). Continuing their research, Keyburn et al. (2010) assessed 44 isolates obtained from chickens with NE from Australia, Europe (Belgium and Denmark) and Canada and 55 ones from healthy Australian and Belgian chickens. As many as 70% of the affected birds were found positive for the NetB toxin, which highly correlated with the presence of netB gene. However, 2 out of the 55 healthy chickens' isolates were carriers of the gene. In addition, it was revealed that netB is highly conserved. However, the gene coding for TpeL toxin was detected in a few NetB-positive isolates from diseased chickens. NetB-negative isolates, obtained from affected birds, failed to produce NE in experimental conditions. Thus, it was proven that NetB is important in pathogenesis.

The first research into the occurrence of the toxin gene (netB) in *C. perfringens* isolates outside Australia was conducted by Martin and Smyth (2009). They inspected 106 American isolates of *C. perfringens* (92 from chickens and 8 from cattle). The netB gene was confirmed in 14 chicken isolates: 7 originated from those with NE, and 7 from non-affected ones, but also in one isolate obtained from a 3-year-old cow with liver abscesses. This is considered to be the first detection of NetB gene in isolates in a non-chicken *C. perfringens* isolate. However, five isolates, each taken from one chicken with NE, were negative for netB gene, as were another 24 isolates recovered from one of these diseased birds. Based on their results, the authors suggest that the importance of NetB for the onset of NE requires additional research and involves assessing the disease-producing capability of both netB-positive strains from healthy chickens, and those netB-negative isolated from diseased birds (Martin and Smyth, 2009). The detection of the netB gene in isolates from healthy birds (in 8.8%) implies that its presence is not sufficient to cause disease, and points to the importance of predisposing factors for the development of NE (Paiva and McElroy, 2014).

It was proven experimentally that TpeL toxin (an acronym which stands for toxin *C. perfringens* large cytotoxin encoded by the *tpeL* gene) may be a significant virulence factor in the development of NE (Coursodon et al., 2012). TpeL belongs to the large clostridial toxins group (LCTs), which is produced by minimum four pathogenic clostridium species: *C. difficile* (TcdA and TcdB

toxins), *C. sordellii* (TcsH and TcsL), *C. novyi* (TcnA) and TpeL *Clostridium perfringens* (TpeL) (Chen and McClane, 2015). TpeL was discovered in *C. perfringens* type C strains, but also in ATCC 3626, a type B strain (Amimoto et al., 2007). Its molecular mass was 191kDa (Amimoto et al., 2017). Some recent research discovered that many type C strains, and nearly all type B strains carry *TpeL* toxin gene, which is often (but not always) located near the *cpb* gene on plasmids of 90 kb or 60 to 65kb (Chen and McClane, 2015). TpeL possesses a glycosyltransferase activity, but is sensitive to trypsin, which is important to the pathogenesis of NE (Amimoto et al., 2007; Chen and McClane, 2015). The cytotoxicity of TpeL was proven on Vero, HeLa and rat pheochromocytoma PC12 cell cultures (Chen and McClane, 2015) and its lethality to mice (Amimoto et al., 2007). This finding implies a potential TpeL contribution to virulence, but this still remains to be proven in animal models (Chen and McClane, 2015). *TpeL* gene was detected in isolates of *C. perfringens* type A possessing also the NetB gene, which suggests that both TpeL-positive strains are associated with NE, although it is considered that the primary role in the pathogenesis is played by NetB toxin (Coursodon et al., 2012; Bailey et al., 2013). TpeL is probably produced also in the guts during clostridium diseases and contributes to type B and C infections in hosts with decreased trypsin levels due to disease, diet or age (Chen and McClane, 2015). It was proven experimentally that TpeL potentiates the effect of other virulence characteristics of NE strains of *C. perfringens* (Coursodon et al., 2012). The virulence of TpeL-positive and -negative *C. perfringens* strains isolated from chicks with NE was examined in healthy birds (Coursodon et al., 2012). Gross lesions typical of NE were observed in all artificially infected birds, but were more prominent in those inoculated with positive strains in comparison with their counterparts inoculated with a negative strain. It was also proven that infection with TpeL-positive strains may produce a disease with a more rapid course and with higher mortality (Coursodon et al., 2012). Although a multiplex PCR has been established for the detection of netB and tpeL genes (Bailey et al., 2013), relatively few bacterial populations have been screened for these two genes (Bailey et al., 2013).

Thus, the lack of extensive epidemiological studies render the global distribution of this newly discovered toxin and its connection with NE unknown (Bailey et al., 2013). It is possible that other virulence factors, such as hydrolytic enzymes and some unidentified toxins may contribute to the pathogenesis of NE (Van Imersseel et al. 2008). Combat against NE in intensive poultry production can be aided by immunization with CPA, NetB or some other proteins, administered conventionally or using recombinant attenuated *Sal-*

*monella* vectors. Inevitably, progress should be based mainly on genomic and proteomic analyses (Coper and Songer, 2009).

## CONCLUDING CONSIDERATION

In the end, it should be kept in mind that modern poultry industry means intensive production whose object is a live system/organism. It is aimed at generating profit, in other words, to gaining maximum quantity of poultry meat in short time at as low cost as possible. However, the production takes place in conditions which are mainly in stark contrast with the lifestyle to which the poultry have been evolutionary adapted to: in closed, overpopulated housing, in the environment with high concentration of causative agents and permanent influence of a battery of factors predisposing to ailments, which are practically impossible to control. Research efforts that aim for casting light on the mechanisms of pathogenesis (which is still to a great extent unclear) are targeted at the choice of potent immunogenes and the improvement in immunoprophylaxis. The question remains open: in those conditions how is it possible to sustain health and the integrity of the intestinal mucosa, the microbiome of the digestive system, and the full potentials of the local gut immunity and the systemic immune response? Thus, we consider that the industrialization of live organisms, in this case of poultry production, will inevitably still be taking its toll: subclinical and acute forms of NE will still occur.

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# AN EXAMPLE OF RATIONAL *DERMANYSSUS GALLINAE* CONTROL – PULCAP

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## Abstract

The irrational control of *Dermanyssus gallinae* ectoparasites (De Geer, 1778) in intensive livestock can cause health problems, economic damage, and can also toxicologically harm the health of humans and the environment. Formulation P 547/17 (Pulcap) is a new generation of inert matter (an oil). In Pulcap formulation and technology, the fulfilment of rigorous criteria for rational control of *D. gallinae* were tested and present in this paper. Those criteria are the following: the safety of the preparation (i.e. the preparation is non-toxic); the feasibility of application of the preparation in facilities, for handling transport cages, on used cages and equipment, as well as in inhabited buildings; the method of application - the preparation is applied by spray method (external application). The aim of the application was also considered. It is to make the control effective, on the condition that eradication is feasible. Then, the conditionality, which imply that the application of this preparation requires fulfilling hygienic conditions, break of production in facilities, a certain temperature and professional approach to it. The efficacy of the preparation is another important criterion. It implies that the clinical results of the application have been monitored in different conditions at different time intervals. It has been proven that there is no resistance to this preparation. The disadvantages of the preparations were also observed. It is the absence of prolonged action on absorbent surfaces, the delay in starting the egg bar and discharging, which increases contamination. The whole program / plan of long-term control has been developed. The cost-effectiveness of the product is the ratio of price and efficiency on an annual level. Eradication is the most economical method of controlling *D. gallinae*, which relieved farmers from all further costs. There is a great

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challenge for each type of *D. gallinae* control, especially the rational one. Reconsidering and further development of the approach of rational control is a way to improve the control of *D. gallinae*, which we want to encourage.

**Key words**: rational control, *Dermanyssus gallinae,* PULCAP

# PRIMER RACIONALNE KONTROLE *DERMANYSSUS GALLINAE* -PULCAP

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## Kratak sadržaj

Neracionalna kontrola ektoparazita *Dermanyssus gallinae* (De Geer, 1778) u intenzivnom živinarstvu prouzrokuje zdravstvene probleme i ekonomske štete, a može i toksikološki da naškodi zdravlju čoveka, živine i životnoj sredini. Za razliku od insekticida, formulacija P 547/17 (Pulcap) je nova generacija inertnih materija, koja spada u grupu netoksičnih jedinjenja a formulacija koja se primenjuje je u vidu vodene emulzije. U cilju racionalne kontrole *D. gallinae* sa P 547/17 (Pulcap) vršena su ispitivanja ispunjenost više kriterijuma koja bi potvrdila efikasnost preparata. Kriterijume koje smo ispitivali bili su sledeći: Bezbednost preparata, odnosno da je preparat netoksičan; Mogućnosti primene u pripremi objekata, za tretiranje transportnih kaveza, polovnih kaveza i opreme, kao i u naseljenim objektima; Vreme primene koje se baziralo na vremenu preventivnog delovanja; Način primene - preparat se aplikuje sprej metodom, što predstavlja spoljašnju primenu; Cilj primene - da suzbijanje bude visoko efikasno, a po sticanju uslovnosti da je moguća eradikacija; zatim uslovnost, što je podrazumevalo da je za primenu preparata neophodno obezbediti higijenske prilike, odmor objekta, određenu temperaturu i stručnu primenu; Efikasnost preparata što podrazumeva da su klinički rezultati primene praćeni u različitim uslovima pripreme objekata pred naseljenje u različitim vremenskim intervalima; Rezistenciju i druge adaptivni mehanizme pri čemu je dokazano da ih preparat ne stvara. Praćeni su i nedostaci preparata i to je izostanak produženog delovanja na upijajućim površinama, zastoj

u pokretanju trake za jaja i izđubravanju, što povećava onečišćenje. Razvijen je ceo program/ plan dugoročne kontrole. Ekonomičnost preparata predstavlja odnos cene i efikasnosti na godišnjem nivou. Eradikacija je najekonomičniji vid kontrole *D. gallinae,* kojom se farmer oslobađa svih daljih troškova. Postoji velik izazov za svaku vrstu kontrole *D. gallinae*, a posebno onu racionalnu. Preispitivanje i dalji razvoj racionalne kontrole, je put unapređenja kontrole *D. gallinae,* koji želimo da podstaknemo.

**Ključne reči:** racionalna kontrola, *Dermanyssus gallinae,* PULCAP

## INTRODUCTION

Non-rational control of the ectoparasite *Dermanyssus gallinae* (De Geer, 1778) in the intensive poultry industry causes health problems and economic loss (Emous, 2005; 2017), and it can cause toxicological harm to human and poultry health and the environment (Giangaspero et al., 2011; Marangi et al., 2012; Giangaspero, 2017). This problem has existed for several decades and it has a high prevalence (Sparagano et al., 2009; Mul et al., 2016), with a tendency of further increase.

## CRITERIA FOR RATIONAL *D. gallinae* CONTROL

According to the World Health Organization (WHO), rational pharmacotherapy requires that "patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements, for an adequate period of time, and at the lowest cost to them and their community". Based on the general definition and specific demands of this issue, we propose the following criteria for rational *D. gallinae* control:

1. 1. Safety
2. Feasibility of application
3. Time of application: preventive or curative approach
4. Method of application
5. Aim of application: suppression or eradication
6. Necessary conditions
7. Efficacy: laboratory and clinical
8. Resistance and other adaptive mechanisms
9. Disadvantages
10. Long-term and comprehensive control plan: the program
11. Cost-effectiveness

The P 547/17 formulation (Pulcap) is a new generation of inert substances, which is applied in the form of water emulsion. It is registered as a product for general use. The criteria of rationality regarding Pulcap formulation and technology are the following:

## *Safety*

Synthetic, neurotoxic chemical compounds - acaricides or insecticides are the predominant type of *D. gallinae* control. The first problem with these compounds is their potential toxicity to humans, animals and the environment.

Unlike insecticides, Pulcap formulation falls into the category of non-toxic compounds. In addition to this, its application focuses on the poultry housing preparation, which prevents any kind of exposure or stress. Only in cases when the necessary conditions have not been met, application is suggested with the poultry inside. In this situation, the formulation's efficacy helps to avoid excessive use. Pulcap formulation and technology fulfils the highest safety criteria.

In general, herbal products are an appropriate choice regarding safety. The safety of SiO2-based products depends on the content of a specific formulation and on the application technology itself.

## *Feasibility of application*

Pulcap formulation is applied in the preparation of (empty, cleaned and disinfected) poultry houses, treatment of poultry transport cages, and treatment of used cages and equipment (Fig 1). If necessary, it can be used in a full house, with poultry inside.

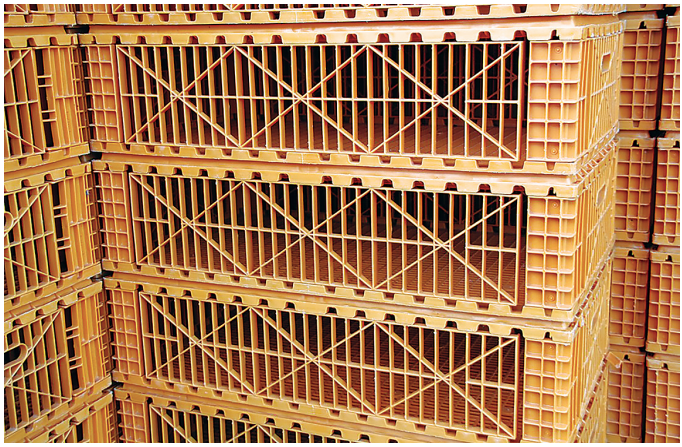

Figure 1. The transport cages for poultry are used for the colonization and emigration flock

Veterinary medicine flurararaner (Exzolt) can be used only for the treatment of poultry, while other insecticides for external use can also be used for housing preparation. Rational application of insecticides on transport cages is problematic due to very demanding conditions for the detection of resistance. The situation is similar with used cages and equipment - if the examination for resistance is not performed on the infested flock before the placement.

Selected $SiO_2$ formulations (combination of powder and liquid form), can be a good choice for housing preparation if the necessary conditions have been met. They are also used in production with a significantly lower effect (Fig 2). They are not appropriate for application on plastic transport cages, while the treatment of used cages and equipment depends on a specific situation. Herbal formulations are prepared primarily for the application through poultry's feed and water.

### *Time of application: preventive or curative approach*

The current concept of application of insecticides and herbal formulations is based on the curative approach. As opposed to this, Pulcap is based on the preventive action, during pre-placement housing preparation (Fig 3). The preventive approach should be chosen because of the following: it prevents exposure (of poultry, eggs, staff) and ensures the safety of the procedure; it maximizes the effect; it prevents damage; it prevents the transmission of *D. gallinae*; it prevents the spreading and development of the disease; it decreases the possibility of resistance development; it is cost-effective; and the flocks are treated only if necessary. The treatment of the flock as the primary concept of *D. gallinae* control is wrong, because it can be successfully prevented (avoided). Instead of biosecurity measures, farmers are offered Q – Perch Vencomatic equipment for active *D. gallinae* control (van de Ven, 2016).

Figure 2. Highly infested flocks with D. gallinae

### *Method of application*

The correct application technology (*D. gallinae* control has been focused only on products) has in most cases been missing in practice so far, thus significantly contributing to the existing negative tendency of Dermanyssosis. Timely procedure and comprehensive and systematic application are necessary.

External application by spraying is the most common method of application. Compared to this, *per os* application has an advantage because it is simple to perform, but at the same time, it is much more complicated regarding safety (which is a much more important criterion), since the formulation is incorporated directly into the poultry's body and, thus, into human food.

Machine application technology has been developed for Pulcap formulation. It includes mounting a structure with nozzles, which systematically applies the water emulsion on the targeted places. This way, a high operability is enabled and three persons can treat the capacity of up to 100,000 layers in the cage system per day (Fig 4). At the ends of batteries, in small farms and other types of layer housing, manual application can be implemented.

Figure 3. Poultry house with enriched caging prepared before the flock placement, using Pulcap technology.

Figure 4. Application of aqueous emulsion formulation Pulcap

## *Aim of application: suppression or eradication*

The current practice is based on *D. gallinae* suppression. Therefore, *D. gallinae* in intensive poultry production persists and spreads, and farmers have perpetual expenses, which constantly rise. *D. gallinae* eradication from the production facilities of the poultry industry is possible and justified for various reasons (Pavlićević et al., 2018a). It provides the highest level of long-term safety and cost-effectiveness in *D. gallinae* control, as well as in the control of infectious diseases. Suppression should only be a temporary phase until the conditions for eradication are created. The *D. gallinae* problem can be solved and it does not have to exist in intensive poultry production. It has been proven that eradication can be achieved with Pulcap technology (Pavlićević et al., 2018b).

## *Necessary conditions*

Application of Pulcap technology requires hygienic conditions, housing downtime (14 days or longer for eradication), temperature above freezing, and professional application. Other approaches in *D.gallinae* control have similar requirements and conditions (to a greater or lesser extent). The essence of the problem is that most often conditions and requirements are not sufficiently specified nor is abiding by them emphasized enough.

## *Efficacy*

Laboratory tests provide important, but limited guidelines for the biological efficacy against *D. gallinae*. It is very important for insecticides to have specification for a concrete infestation and to be based on up-to-date information. Resistance development has eliminated most of the insecticides used so far, so a lot of older data on efficacy testing is no longer relevant. The latest insecticide is fluralaner (Exzolt). It is used to treat poultry and is applied through drinking water twice over a period of 7 days. Acaricide efficacy of fluralaner applied in this way over 15 days is between 99.3% and 100% (Brauneis et al., 2017). It is estimated that this veterinary medicine achieves suppression of over 90% in an average period of 8 months (56 – 238 days) (Thomas et al., 2017).

The efficacy of different SiO2-based formulations varies significantly (Maurer et al., 2009; Schulz, 2014; Pavlićević et al., 2018c). For this reason it is necessary to choose the formulation based on efficacy tests. Moreover, SiO2-based formulations have extremely demanding application, small acaricide capacity per surface unit and slow effect, so even the lethally exposed mites can lay fertile eggs. Their action is affected by dirt and humidity (Pavlićević et al., 2017, 2018b). Their application is optimized in the control program, by combined application of powder and liquid forms in an empty poultry house and with housing downtime in the temperature conditions when mites are active (Pavlićević et al., 2017, 2018b).

The most important disadvantages of SiO2-based formulations are eliminated by the new generation of inert substances - Pulcap. It is a water emulsion of inert oils, it has good applicability, distribution, a quicker effect on the exposed *D. gallinae*, higher mortality per surface unit, and extremely long prolonged effect on unabsorbent surfaces. Clinical results with all the conditions fully met, after the application of 20% Pulcap water emulsion, were negative in 8 poultry houses with cage rearing system (a total capacity of 144,000 chicks) during 6 months, over the period after rearing two flocks in each (the

observation is ongoing) (Fig 5). Another example was in partly met conditions in 7 poultry houses with the capacity of 241,360 layers, in a cage system (conventional and enriched). In addition to the housing preparation with 20% Pulcap water emulsion, over the production period of one year, another treatment with 10% water emulsion was necessary. In three houses with the total capacity of 103,000 hens, only one preparatory treatment was sufficient for the suppression effect over the production period of one year. However, in cases when conditions were fully met, there are examples of complete eradication from the production facilities (so far confirmed in 2 houses with conventional cage system, a total capacity of 6,000 hens). In the case of application in a full house, one treatment with 10% Pulcap water emulsion provides 3 to 4 months of suppression on average. However, two consecutive treatments increase the efficacy in a full house (up to 5-10 months, according to the experience so far). Based on the recorded results, Pulcap technology belongs to the efficient methods of *D. gallinae* control. Nevertheless, we believe that the technology has not been optimized yet and that even higher efficacy than the one recorded so far can be achieved. Optimization tests are ongoing, and the results are expected by the end of 2020.

Figure 5. The cages for rearing hens treated technology Pulcap

During and after the application of phytorepellents, feeding and reproduction of *D. gallinae* were reduced (Puvača et al., 2016). The problem is that the number of mites per hen still increases over time, so the numbers neutralize the positive effect achieved so far. In addition to this, as a result of the application of phytorepellents on poultry, the mites increasingly attack farm staff. Due to the (significant) lack of cidal or real repelling effect (which would stop the reproduction completely), phytorepellents are actually only partial repellents. Such characteristics qualify them primarily as an auxiliary product in *D. gallinae* control. A good choice of this product can be justified if it is necessary to maintain a tolerable, cost-effective level of infestation at the end of the flock's production period, until the flock is removed from the house.

### *Resistance and other adaptive mechanisms*

Another problem of predominantly treating *D. gallinae* with insecticides is resistance (Abbas, 2014; Pavlićević et al., 2016). So far there has not been a regular monitoring of resistance in *D. gallinae* control. In that aspect, acaricide application in *D. gallinae* control has so far not been rational. In the application of SiO2 formulations, adaptations in *D. gallinae* behavior have been recorded - they moved to places which are not accessible for application.

Unlike acaricides, it is believed that the development of chemoresistance to Pulcap formulation is not possible, and due to a better distribution neither is the development of other adaptive mechanisms which have been recorded in SiO2 formulations (Fig 6). Efficacy of Pulcap formulation and technology will not weaken over time.

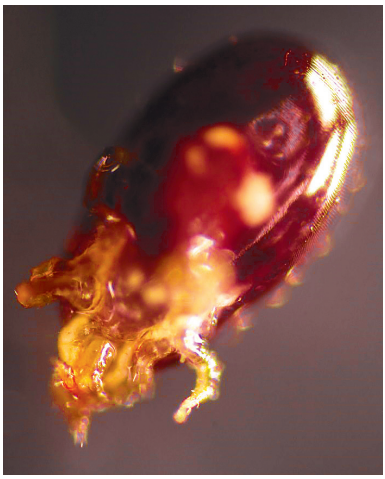

Figure 6. The physical effect of the Pulcap formulation on D. gallinae

### *Disadvantages*

Pulcap formulation does not have prolonged effect on absorbent surfaces, such as a concrete floor. This flaw requires repeated application, so that the subsequently exposed mites can also be affected. Application of Pulcap water emulsion can cause intermittent movement of conveyor and manure belts, which can be fixed by applying adsorptive powder (Fig 7). Cages and equipment get dirty to a somewhat greater extent, but hygienic conditions can be maintained.

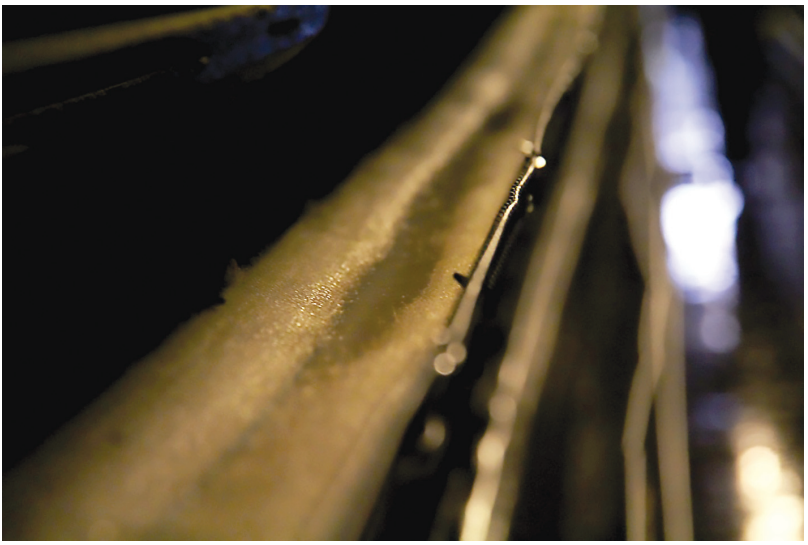

Figure 7. A tray for the transport of eggs exposed to aqueous emulsion of the Pulcap formulation

The disadvantages of SiO2 formulations and technology are the following: complex application and equipment, limited distribution, abrasive effect on cages and equipment, and dust in the application of powder forms (Pavlićević et al., 2018c).

### *Long-term control plan: the program*

In addition to the application technology, we have also designed a complete program based on Pulcap formulation and control technology. The program provides a comprehensive approach and maximizes the principles of preventive medicine and rational control through the short-term goal of highly ef-

ficient and safe suppression and the long-term goal of *D. gallinae* eradication and introduction of biosecurity measures (Pavlićević et al., 2018b). Another important element of the program is the environment and simplification of its conditions (Pavlićević et al., 2019). The program will produce its full effect only when it is implemented systematically, on a wider area, horizontally and vertically (parent flocks, rearing, production), which requires institutional support. For new and non-infested farms and poultry houses, the program is based on biosecurity - prevention of mite introduction.

### *Cost-effectiveness*

The cost of a product and technology can be correctly estimated only through its cost-effectiveness ratio. A proposed estimation of products and methods for *D. gallinae* control is the annual expenditure (during the regular flock production period). The control program, which enables eradication from the production facilities and introduction of biosecurity measures, is the most cost-effective type of *D. gallinae* control, which eliminates any further expenditures for the farmer.

### *Other*

Adequate prerequisites for the rational control are detection and monitoring are necessary because they provide a relevant insight into the presence of *D. gallinae* infestation, its intensity and extensity (Pavlićević et al., 2007, 2018b).

The rational control must be up-to-date. Therefore, it is conditioned by regular periodic comparative assessments of formulations and methods for control, as well as providing timely information for experts and farmers.

## CONCLUSIONS

Having analyzed the 11 criteria of rational control and compared it to the most important current solutions, we found that Pulcap formulation and technology is an example of rational control of *D. gallinae*. The use of non-toxic substances in prevention, with proven high efficacy creates the necessary basis for the fundamental improvement of *D. gallinae* control. There are disadvantages, which are important to be minimized with further development and, as much as possible, to be avoided or totally eliminated in the end.

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# THE INFLUENCE OF LIQUID CALCIUM SOURCE ON EGGSHELL QUALITY

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## Abstract

It is estimated that the world's population will enlarge by 25% by the middle of this century, resulting in the food production increase by at least 60%. Intensifying egg production is one of the most affordable solutions to provide animal protein. Among many other efforts to improve the quality of eggs, special attention is paid to attempts to provide a better endurance and strength of eggshell, due to the fact that the production of eggs with broken, cracked or soft shells incurs significant economic losses. One of the most important factors to achieve this goal is careful adjustment of calcium in the diet of laying hens, but the nutritional role of calcium is closely linked to that of phosphorus and the effect of vitamin D.

The aim of the experiment was to determine whether two different nutritional supplements of calcium, applied in drinking water of laying hens for two weeks have any significant influence on the shell egg quality. Statistically very significant difference in egg production was observed in the treatment with the product containing calcium and phosphorus. On the other hand, the product consisting of calcium and vitamin D3 had no beneficial impact on the egg parameters.

Based on the obtained results and literature data, it can be concluded that the amount and source of calcium in the diet of laying hens is a very complex and not fully solved issue. Therefore, especially keeping in mind the duration of the experiment, additional research is needed on this subject.

**Key words***:* calcium, eggshell, laying hens, liquid supplement

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# UTICAJ TEČNOG DODATKA KALCIJUMA NA KVALITET LJUSKE JAJETA

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## Kratak sadržaj

Procenjuje se da će se svetska populacija uvećati za 25% do sredine ovog veka, te da će do tada i potreba za proizvodnjom hrane porasti za 60%. Intenziviranje proizvodnje jaja je jedno od najpristupačnijih rešenja za obezbeđivanje neophodnih proteina životinjskog porekla. Među mnogim drugim naporima da se poboljša kvalitet jaja, posebna pažnja se posvećuje nastojanju da se unapredi izdržljivost i jačina ljuske jajeta, zbog činjenice da je proizvodnja jaja sa slomljenom, napuklom ili mekanom ljuskom uzrok značajnih ekonomskih gubitaka. Jedan od najvažnijih faktora za postizanje ovog cilja je pažljivo balansiranje kalcijuma u ishrani koka nosilja, kao i izbor odgovarajućeg izvora mineralnih materija.

Cilj eksperimenta bio je da se utvrdi imaju li dva različita nutritivna dodatka kalcijuma, primenjeni u vodi za piće nosilja tokom dve nedelje, značajnog uticaja na kvalitet ljuske jaja. U grupi nosilja u čiju je vodu za piće dodat proizvod koji sadrži kalcijum i fosfor, u odnosu na kontrolnu grupu, uočene su statistički značajne razlike u broju proizvedenih jaja. S druge strane, proizvod koji se sastoji od kalcijuma i vitamina D3 nije imao uticaja na ispitivane parametre ljuske jaja.

Na osnovu dobijenih rezultata i podataka iz literature, može da se zaključi da je pitanje količine i izvora kalcijuma u ishrani nosilja veoma složen i ne u potpunosti rešen problem. Naročito imajući na umu dužinu trajanja eksperimenta, neophodna su dodatna istraživanja na ovu temu.

**Ključne reči**: kalcijum, ljuska jaja, nosilje, tečni dodatak

## INTRODUCTION

It is estimated that until 2050 the world's population will enlarge by 25%, and the demand for increase in food production will grow by at least 60% (FAOSTAT, 2013). Eggs constitute one of the most affordable source of animal protein available, so the number of laying flocks is rapidly increasing in developing countries. In Europe, the priority is to increase egg production by breed-

ing for increased persistency in lay and stability in egg quality so that the laying cycle of commercial flocks can be extended to 90–100 weeks (Bain et al., 2016).

A number of factors have an impact on the quality of eggs, such as breed and age of laying hens, inheritance, feed, the environmental conditions or diseases. Endurance and strength of eggshells require special attention as the production of eggs with broken, cracked or soft shells incurs significant economic losses. Eggshells of laying hens are highly organized mineral structures built of spherical calcite crystals deposited on the outer surface of the protein membrane, around egg white. The crystals of calcium carbonate are formed by crystallization from secreted supersaturated solution in the distal part of the oviducts. It takes about 20 hours for the formation of an eggshell, which indicates a large demand for a constant supply of the necessary amounts of calcium (Vitorovic et al., 2004).

During the laying period, the first challenge is to adjust the energy and protein requirements to optimize egg output and to carefully control body weight. The crude protein concentration and amino acids in the layer diet are also important, methionine being the main limiting amino acid. The provision of insufficient dietary calcium during the rearing or laying period has an adverse effect on both eggshell quality and bone strength (Bain et al., 2016). The laying hens requirement for dietary calcium within the diet for different ages is in the range from 0.9 to 1.2% during the growth period of the pullet, increasing to 2 to 2.5% just prior to the onset of lay and 3.5 to 4.5% once a lay is established (Bouvarel *et al.,* 2011).

Shell formation takes place mainly during the night when consumption is very low. Therefore, it is recommended to replace a portion of a fine pulverized calcium source in the diet with a larger particle size limestone, which takes more time to dissolve in the digestive tract, and thus presents calcium available overnight (Pavlovski et al., 2003). It is also recommended to implement the so-called night diet, when hens are stimulated to feed intake by periodic lighting.

Having all the above mentioned in mind, the aim of this study was to determine the influence of two nutritive supplements, as additional calcium sources administered in the drinking water on the quality of the eggshell.

## MATERIALS AND METHODS

The experiment was conducted on 34200 Lohmann Brown laying hens, 78 weeks of age. All of them were fed with a mixture of standard ingredient structure as shown in the Table 1, and the chemical composition shown in the Table 2.

Table 1. Ingredient composition of feed mixture

| Ingredients | Composition [%] |
|---|---|
| Maize | 56.00 |
| Sunflower meal | 12.50 |
| Soybean cake | 8.75 |
| Soybean oil | 0.75 |
| Cereal meal | 7.50 |
| Mineral chalk | 3.75 |
| Grit 2-4 mm | 6.25 |
| Yeast | 2.50 |
| Premix | 2.00 |
| TOTAL | 100.00 |

Premix composition [%] was the following: Cereal flour 46.393, Sodium bicarbonate 12.500, Monocalcium phosphate 12.386, Sodium chloride 9.508, Lysine 7.962, Methionine 4.968, Hepatron 95%/Choline 1.579, Mangan sulphate 1.094, Iron sulphate 0.968, Zinc sulphate 0.877, Axtra XB 201 laying hens 0.500, Vit. E 50 S 0.400, Copper sulphate 0.160, Axtra phytase TPT 0.150, Vit. B3 Niacin 0.147, Microgran Se 1% 0.125, Vit. B5 98% Pantothenic acid 0.061, Microgran I 10% 0.050, Vit. A 1.000.000 IU/g 0.050, Vit. K 51% 0.029, Vit. B2 80% 0.025, Vit. D3 500.000 IU/g 0.025, Vit. B6 99% 0.020, Vit. Biotin 2% 0.012, Vit. B12 1% 0.008 and Vit. folic acid 80 0.002.

Table 2. Chemical composition of feed mixture

| Component | Units | Value | Component | Units | Value |
|---|---|---|---|---|---|
| MEpoultry | kcal | 2734.665 | Vit E | IE/g | 0.040 |
| MEpoultry | MJ | 11.437 | Vit K | mg/kg | 3.000 |
| Crude protein | g/kg | 144.759 | Vit B1 | mg/kg | 3.250 |
| Crude fat | g/kg | 35.617 | Vit B2 | mg/kg | 5.000 |
| Crude ash | g/kg | 125.071 | Vit B6 | mg/kg | 5.000 |
| Crude fiber | g/kg | 46.961 | Vit B12 | µg/kg | 15.000 |
| Calcium | g/kg | 40.310 | Niacine | mg/kg | 40.000 |
| Phosphor | g/kg | 5.905 | FolicAcid | mg/kg | 0.750 |
| AvPhosphrPoultr | g/kg | 3.300 | Biotin | µg/kg | 50.000 |
| Sodium | g/kg | 1.700 | Betaine | mg/kg | 300.000 |
| Cl | g/kg | 1.872 | Fe | mg/kg | 60.000 |
| Lysine | g/kg | 7.500 | Cu | mg/kg | 8.000 |
| Methionine | g/kg | 3.748 | Zn | mg/kg | 60.000 |
| Methionine+Cyst | g/kg | 6.400 | Mn | mg/kg | 70.000 |
| Threonine | g/kg | 5.484 | I | mg/kg | 1.000 |
| Tryptophane | g/kg | 1.545 | Se | mg/kg | 0.250 |
| Vit A | IE/g | 10.000 | Ca-pan-thotenate | mg/kg | 12.000 |
| Vit D3 | IE/g | 2.500 | | | |

Cage rearing facility consisted of three batteries, each containing 184 cages. The layers were thus divided into 3 experimental groups consisting of 11,400 individuals per each (R, C and L), which were of uniform performance and egg production characteristics. Every group had its own dispenser of drinking water. During 8 hours a day an experimental group R was given a product which contained calcium 5 g/l and phosphorus 144 g/l, in the dosage of 1 l per 1000 l of drinking water. At the same time experimental group L received another product containing calcium 75 g/l and vitamin D3 300 000 IU/l, in a dosage of 1 l per 1000 l of drinking water. For the control group C only pure water was provided. As different additives were used, it was not the goal to make a comparison between them, but to gain, in practical farm conditions, a quick insight into their impact in relation to the control group.

The experiment lasted for 15 days. During that period, there were five samplings of eggs, always within the same central part of the hall. The same number of eggs (30 eggs of A and 30 eggs of SS class) were taken from each group separately. Out of every 30 eggs, 14 were selected for weight measurements, with 2 lightest and 2 heaviest eggs discarded from statistics. The remaining 10 eggs were measured for the eggshell thickness using a micrometer after rinsing the shell in water to remove any adhering albumen. Later on, in order to determine the eggshell weight, drying at 105 °C for two hours was performed with the shell membrane intact. This was followed by weighing on an analytical scale. The percentage of eggshell breakage was also recorded as a percentage of the cracked eggs in relation to the number of whole eggs.

Statistical analysis was done using Microsoft Excel 2007, while the differences in measurements between the groups were tested by paired two-tailed t-test and ANOVA Two-Factor.

## RESULTS AND DISCUSSION

The obtained results are given for each parameter separately as follows: The number of produced and cracked eggs (Table 3), the percentage of eggshell breakage (Table 4), Egg weight (Table 5), Eggshell weight (Table 6) and Eggshell thickness (Table 7).

Table 3. Number of eggs / Number of cracked eggs

| Group | 1st day | 8th day | 10th day | 12th day | 15th day |
|---|---|---|---|---|---|
| R | 9964/ 277 | 10386/ 320 | 10185/ 279 | 10114/ 290 | 11180/ 356 |
| L | 9916 / 272 | 10138/ 240 | 9971 / 286 | 9919 / 281 | 10921/ 320 |
| C | 9927 / 272 | 10132 / 275 | 9886 / 310 | 9780 / 305 | 10521/ 343 |

Table 4. Percentage of eggshell breakage [%]

| Group | 1tst day | 8th day | 10th day | 12th day | 15th day |
|---|---|---|---|---|---|
| R | 2.78 | 3.08 | 2.74 | 2.87 | 3.18 |
| C | 2.74 | 2.36 | 2.86 | 2.83 | 2.93 |
| L | 2.74 | 2.71 | 3.13 | 3.12 | 3.26 |

Table 5. Average egg weight [g]

| Group | Egg class | 1st day | 8th day | 10th day | 12th day | 15th day |
|---|---|---|---|---|---|---|
| R | A | 62.11±0.87 | 62.96±1.15 | 61.70±1.43 | 62.81±1.02 | 63.14±0.91 |
| | SS | 71.78±0.75 | 72.23±0.88 | 72.49±0.75 | 72.96±0.75 | 73.08±0.88 |
| C | A | 62.17±1.14 | 62.87±1.28 | 61.57±0.93 | 63.38±1.09 | 63.59±1.01 |
| | SS | 71.90±0.74 | 72.32±0.87 | 72.64±0.68 | 72.29±0.95 | 73.29±0.73 |
| L | A | 62.32±1.29 | 62.04±1.16 | 61.42±2.18 | 62.95±0.86 | 62.63±1.51 |
| | SS | 71.92±0.97 | 72.62±0.73 | 73.59±1.19 | 73.06±1.02 | 72.08±0.45 |

Table 6. Average eggshell weight [g]

| Group | Egg class | 1st day | 8th day | 10th day | 12th day | 15th day |
|---|---|---|---|---|---|---|
| R | A | 6.523±0.712 | 6.919±0.787 | 6.586±0.580 | 6.618±0.454 | 6.705±0.633 |
| | SS | 7.124±0.735 | 7.151±0.672 | 7.204±0.953 | 7.501±0.557 | 7.098±0.730 |
| C | A | 6.395±0.354 | 6.939±0.606 | 6.535±0.344 | 6.911±0.549 | 6.731±0.512 |
| | SS | 7.375±0.461 | 7.589±0.458 | 7.465±0.654 | 6.992±0.559 | 7.186±0.675 |
| L | A | 6.491±0.785 | 7.002±0.559 | 7.066±0.569 | 6.790±0.865 | 6.623±0.585 |
| | SS | 7.150±0.670 | 7.671±0.615 | 7.770±0.700 | 7.232±0.635 | 7.332±0.557 |

Table 7. Average eggshell thickness [mm]

| Group | Egg class | 1st day | 8th day | 10th day | 12th day | 15th day |
|---|---|---|---|---|---|---|
| R | A | 0.351±0.037 | 0.362±0.038 | 0.351±0.049 | 0.339±0.023 | 0.361±0.032 |
| | SS | 0.353±0.039 | 0.356±0.039 | 0.333±0.037 | 0.342±0.020 | 0.355±0.033 |
| C | A | 0.345±0.030 | 0.349±0.042 | 0.329±0.023 | 0.356±0.025 | 0.355±0.023 |
| | SS | 0.351±0.028 | 0.364±0.033 | 0.331±0.040 | 0.324±0.021 | 0.358±0.030 |
| L | A | 0.349±0.028 | 0.365±0.026 | 0.368±0.028 | 0.356±0.034 | 0.356±0.014 |
| | SS | 0.350±0.026 | 0.368±0.030 | 0.348±0.021 | 0.364±0.025 | 0.354±0.017 |

These tables show that there were no statistically significant differences in eggshell quality parameters between the experimental groups. The only parameter that differed significantly comparing to the control group (< 0.01) was the number of eggs in the group R, which was given product containing calcium and phosphorus. Dissimilarity of all other results had no statistical importance.

Some authors, like Tunc and Cufadar (2015), who investigated influence of dietary large calcium sources (limestone, oyster shell and egg shell) on performance and eggshell quality parameters in laying hens observed no effect. However, the diet used in their experiment, containing at least 50% large calcium sources, had positive effect on mineral contents of tibia. Also, in earlier research of Keshavarz and Nakajima (1993) no benefit of adding more calcium to limit the age deterioration in shell quality was determined, as well as no influence of such dietary treatments on egg production. This could be due to some other factors like breed and age of laying hens, inheritance and the environmental conditions, which could be dominant. Increasing the dietary level of Ca without beneficial effects on shell quality indicate that the National Research Council (NRC, 1994) estimation of calcium (Ca) requirement of 3.25 g per hen per day is adequate for optimum shell formation.

On the other hand, experiment performed by Zhang et al. (2017), which lasted for 10 weeks, showed that aged non-molted laying hens (77 weeks) or older molted second cycle layers (94 weeks) require lower calcium solubility and higher calcium intake compared to relative younger laying hens (36 weeks old) to maximize shell quality and bone status. The amounts 3.94 - 4.89 g of Ca intake for a hen per day from calcium carbonate source with a solubility range of 30.1-39.8% was recommended for older layers. Therefore, according to these authors, the NRC recommendation might not be sufficient to support the performance variables such as egg shell quality and bone status in older non-molted laying hens, or older molted second cycle laying hens. Pavlovski

et al. (2003) and Vitorovic et al. (2004) similarly suggested usage of limestone of larger particle size to improve eggshell quality of laying hens, while Wang et al. (2013) had the same conclusion for ducks.

Sharma et al. (2009) used liquid additional supplement source of calcium and phosphorus in their experiment and showed that overall egg and shell quality was improved in correlation with the activity of herbal constituents of the products owing to mineralization properties. Jadhav et al. (2010) also proved that supplementation of the same liquid calcium and phosphorus source was efficacious in enhancing bioavailability of these minerals, thereby improving overall performance and bone mineralization in broilers.

During two weeks of our experiment, additional calcium from liquid source given through drinking water, especially supplement with calcium and vitamin D3 in group L, had no significant impact on the examined parameters. It possibly means that the maximal exploitation of hens, in these circumstances, was achieved through adequate nutrition. In the used complete feed all nutrients were balanced and aligned with the needs of Lohmann Brown breed, while feed calcium was at the level of 3.95%. On this issue McDowell (2017) emphasized that the usage of liquid supplements to provide minerals might not provide enough calcium due to the solubility problems. Also, the duration of the experiment of only two weeks is a possible limiting factor.

Amino acids, in particular methionine and lysine (Bain et al., 2016), as well as fatty polyunsaturated linoleic acid are also important for adequate egg production. Certain improvement in the trial was observed in the treatment with the product containing calcium and phosphorus, which indicates the significance of both minerals and demonstrates the importance of balancing Ca-P ratio in the diet of laying hens (Sefer and Sinovec, 2008). The establishment of Ca and P requirements of commercial layers is a continuous challenge for poultry nutritionists and egg producers as the needs for these two minerals seem to constantly change (Pelicia et al., 2009).

Two-week experiment seems to be a short time to gain a quick insight into the impact of the applied supplements within the farm conditions and in limited material possibilities, compared to the five-week trial carried out by Jadhav et al. (2010). In order to get a clearer picture and deeper understanding of obtained results, the continuity of investigations by extending the experiment, or by examining some other sources and diet regimens of laying hens are required. Also, as no significant differences in effects of two additives of different composition were found, this research limitation has no influence on final conclusions, but affirms the need to engage these complex facts in further investigations as well.

## CONCLUSION

Based on the obtained results and literature data, it could be concluded that the amount and source of calcium in the diet of laying hens is a very complex and still not fully solved issue. Many factors influence the quality of eggshell. Taking into account significant economic losses incurred by the production of eggs with broken, cracked or soft shells, as well as demand to increase food production for 60% by the middle of this century, it is obvious that all research efforts for improvement in breeding and production of laying hens are necessary and important. The extension of the research is required and the topic of this paper should also be addressed.

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- Full name of the institutions should be given below the list of authors (the following order should be considered: university or organization/institution, department, city, and country for all authors).
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Headings in the paper are: **INTRODUCTION, MATERIAL and METHODS, RESULTS, DISCUSSION (or RESULTS and DISCUSSION for Short Communications), CONCLUSION and REFERENCES**. For Review articles instead M&M, Results and Discussion section, the Main text section should subdivided by different subheadings describing topics presented/discussed/analysed. For Case studies this part could be the same or replaced by Case presentation part with or without different subheadings.

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**MATERIAL and METHODS**. Here describe the conditions in the experiment, name the used methods, material and animals. The Ethical statement (or description why ethical statement is not applicable) should be provided at the beginning of the section. This section includes, as appropriate, a description of study design, experimental animals or data about the samples used, analytical methods and statistical analyses. Identify the methods and procedures in sufficient details to allow others to reproduce the study. If methods are widely known, they are not described, but appropriate references must be cited. For new methods, the detailed protocols for the method should be included. Authors must provide references for established methods

including statistical methods. Specify any general computer program used. Identify all drugs and chemicals used with generic or chemical names, doses and route of administration. For diagnostic kits/reagents and instruments used in the study provide manufacturer, product number, city and country where applicable.

**RESULTS**. Results are presented in a logical order and in parallel with the Methods (for every Method, there should be a Result), using text descriptions and Tables and/or Figures without duplicating the results between these formats. To enhance clarity, this section can be divided into subsections, each with a concise subheading in italics and which provides details of findings that are required to support the conclusions made in the manuscript.

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**ACKNOWLEDGEMENT.** The source of funding for the study should be stated in this section. Also, those who have made a substantial contribution to the study in terms of design, execution, analysis or manuscript drafting/revision but do not fit the criteria for authorship should be mentioned in this section. It is the responsibility of the Authors to ensure that those being acknowledged have agreed to being named in this part.

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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.
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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.

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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**

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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.**

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