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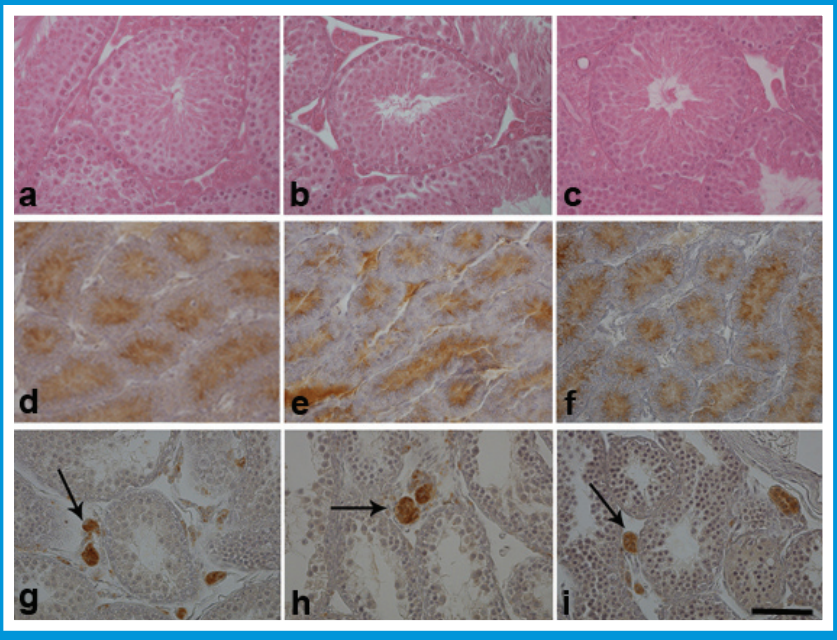
# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK

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**Review Article**

# PYRETHROIDS INFLUENCE ON FISH

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**Summary:** Pyrethroids belong to the most commonly used pesticides worldwide. Their massive expansion is a threat to the natural environment including the aquatic ecosystems. Although pyrethroids are rapidly degraded in soil and plants, they are extremely toxic to fish because of fish high sensitivity to them.
Pyrethroids are divided by characteristic into type I and type II. Both types cause similar neurological symptoms. They affect sodium channels of nerve filaments and type II pyrethroids even affect chloride and calcium channels. Critical in fish pyrethroid intoxication is slower elimination than in birds and mammals. Pyrethroids are absorbed by fish gills readily. After distribution to bile, liver, kidney and red blood cells, they are metabolized by hydrolysis, hydroxylation and conjugation to glucuronides and sulphates. Disorders of movement and breathing during acute poisoning are followed by death. Chronic effects of pyrethroids induce behaviour changes, blood profile changes, histopathological changes, decreased growth, immune system effects and endocrine effects. Both types of toxicity reduce reproductive potential. Toxicity of pyrethroids depends on many external and internal factors.

**Key words:** pyrethroids; fish; neurotoxicity; sensitivity; physiological disturbances

**Abbreviations & Units:** ALP - alkaline phosphatase; ALT - alanine transaminase; AST - aspartate transaminase; ATP - adenosine triphosphate; CA - carbonic anhydrase; CK - creatine kinase; EPA - Environmental Protection Agency; GABA - gamma-aminobutyric acid; GDH – glutamate dehydrogenase; Hb - haemoglobin; HSP - heat shock protein; LDH - lactate dehydrogenase; MCH - mean cell haemoglobin; MCHC - mean corpuscular hemoglobin concentration; MCV - mean cell volume; mRNA - messenger ribonucleic acid; PCV – packed cell volume, haematocrit; PGF2α – F-type prostaglandin; RBC - erythrocyte counts

## Introduction

Pyrethroids are synthetic analogues of the natural pyrethrins, extracts of the ornamental *Chrysantemum cinerariaefolium* and its related species. Pyrethrins had been used for decades for control of insects. They were selective, safe and had short half lives. Although they were acutely toxic to fish, very few accidental poisoning occured because they were not registered for aquatic use and they seldom had enough persistence to reach water from normal application (1).

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The 1st generation of pyrethroids was developed in the 1960s, the 2nd generation was developed in 1970s. Many of pyrethroids have been produced with improved physical properties (involatility, lipophilicity) and greater insecticidal activity (knockdown) since then (2). Pyrethroids disrupt the insect nervous system and this determines them to protect food grains and other agricultural products against pests. They began to be used as ectoparasiticides in veterinary and human medicine too (3, 4). They have replaced natural pyrethrins especially due to their better photostability gradually. Pyrethroids use has increased rapidly in the past three decades. Pyrethroids are thermostable and photostable, slightly soluble in

water and highly soluble in fats. The presence of halogens in some pyrethroids contributes to the greater persistence and provides better residual activity against insect together with higher potential negative effects on the environment (5).

## Classification of pyrethroids

Pyrethroids are divided into type I and type II, based on their structure, chemical and neurophysiological properties and toxicological action. Type I pyrethroids are without a cyano moiety at the α-position (i.e. permethrin, bifenthrin, allethrin, tetramethrin, resmethrin, phenothrin, bioresmethrin, etofenprox, prallethrin, tefluthrin), while type II pyrethroids have an α-cyano moiety at the benzylic carbon of the alcohol portion of the ester (i.e. cypermethrin, cyfluthrin, deltamethrin, cyphenothrin, flumethrin, cycloprothrin, fenvalerate, fluvalinate). Type II pyrethroids are more effective (6). All pyrethroids affect the sodium channels of nerve filaments. They extend time of opening and closing of sodium channels and extend their depolarisation phase. Moreover, type II pyrethroids affect the GABA receptors in the nerve filaments and affect chloride and calcium channels (6-9). Type I pyrethroids cause a type I poisoning called *"T syndrome"*, whereas type II pyrethroids induce a type II poisoning, known as *"CS syndrome"* in mammals (2). T- syndrome mainly includes symptoms like aggressive sparing behaviour, increased sensitivity to external stimuli, fine tremors, prostration, coarse body tremor, increase of body temperature. Pyrethroids that induce a "choreoathetosis with salivation" response are called CS-syndrome pyrethroids and result in a broader range of toxic events due enhanced neurotransmitter release. Their main symptoms are: chewing, profuse salivation, pawing and burrowing, coarse body tremor, increased startle response, abnormal locomotion of posterior limbs, sinuous writhing (choreoathetosis) and clonic and tonic seizures (7). They cause cardiac contractions (3).

Summarized all pyrethroids interfere with nerve cell function by interacting with ion channels. Pyrethroids also modulate the release of acetylcholinesterase in the brain (10) and can inhibit ATP-ases (11). They can disrupt hormon-releated functions. But their effects on the endocrine system are not described uniformly (12).

## Presence in the aquatic environment

Pyrethroids are absent in natural water normally. They may contaminate aquatic ecosystems as pollutants, because they are an important group of pesticides. The contamination of surface waters by pesticides used in agriculture is a problem of worldwide importance (10, 13). Ecological catastrophes following application of deltamethrin for mosquito control have already been in 1991 and 1995. Deltamethrin exposure have been one of main causes of massive eel (*Anguilla Anguilla L.*) devastation in Lake Balaton, Hungary (14). Pesticides are also very important in veterinary medicine as ectoparasiticides. They are popular due to their strong and extended insecticidal and simultaneously acaricidal effects. Pyrethroids are also used as antiparasitic drugs in human medicine and they are used extensively in urban settings to control several medically important insects that vector diseases. In aquaculture, pyrethroids are applied to control some parasitic diseases caused by, for example, *Lepeophtherius salmonis* or other sea lice in salmon farming. These products mainly based on deltamethrin are used in Scandinavian countries or Canada (15, 16). In addition to the recent increased interest in introduction of using deltamethrin in warm waters too, there are encouraging therapeutic results against isopoda with no side effects on the sea bass (*Dicentrarchus labrax L.*) (17).

Aquatic organisms can be affected by pesticides during their improper application or improper handling. Pesticides can get into the water directly due to the incorrect application. They can get into the water during the disposal of unused residues or due to accidents during transport. Pesticides also can get into the water indirectly after running off from surrounding treated products (18). The residues of cypermethrin have been widely detected in water and sediment samples from streams and rivers draining major agricultural districts (19).

## Toxicity in the aquatic environment

Pyrethroids are fairly rapidly degraded in soil and plants in the environment (2). Pyrethroids induce rapid onset of poisoning symptoms but persist only for a short time in the water column due to ability of adsorption by organic matter and deg-

radation (20). The major degradation processes are ester hydrolysis and oxidation at various sites of the molecule. Pyrethroids have high hydrophobicity and they are rapidly and strongly adsorbed into particulate material (21). The pyrethroids are strongly adsorbed on soil and sediments. Pyrethroids are widely recognized as being strongly lipophilic, and thus highly hydrophobic (21-23), adsorbing almost exclusively to organic carbon molecules in water sediment slurries within 24 hours (24). Furthermore, pyrethroids have shorter chemical half-lives than their organophosphate predecessors, ranging from several days (22) to around one month in aerobic sediments (25). Sediment organic carbon plays a critical factor in determining the bioavailability of a given pyrethroid in a particular aquatic system, and accordingly, the pyrethroid's potential toxic effects (24). Microbial biodegradation of pyrethroids in aquatic system (in the sediment and water column) has been acknowledged to play an important role in the degradability and the persistence of the residues (26).

## Fish sensitivity

Pyrethroids have been shown to be up to 1000 times more toxic to fish than to mammals and birds at comparable concentrations (5, 27). Fish sensitivity to pyrethroids may be explained by their relatively slow metabolism and slow elimination of these compounds (7, 28). It may be explained as a result of exposure of toxicokinetic (i.e. absorption, biotransformation, distribution and elimination) and toxicodynamic (i.e. biochemical and physiological effects) factors (7). Unlike most animals, in which pyrethroids have a short life and are readily metabolized, fish are reported to be deficient in enzymes that hydrolyze these insecticides (1, 29-31).

The hypersensitivity of fish to pyrethroid intoxication is due partly to species specific differences in pyrethroid metabolism, but second important factor is higher sensitivity of the piscine nervous system to these pesticides. Fish brain seems to be more susceptible to pyrethroids than mammal and bird brains are (1, 32). The third factor is route of exposure. Pyrethroids are absorbed directly via the gills into the blood stream (31).

Pyrethroids are inhibitors for fish carbonic anhydrase enzymes, and might cause undesirable results by disrupting acid–base regulation as well as salt transport. The most potent inhibitor is deltamethrin. The most affected CA enzymes are in muscle tissue and the lowest inhibition of CA enzymes is in liver tissue (33).

## Types of poisononig

### *Acute toxicity*

Acute toxicity is defined as a significant reduction in survival of the exposed organisms within a relatively short time and is expressed as the species specific median lethal concentration (LC50) (12). The value 96 h LC50 is under 10μg/L in fish generally. Salmonid species are more susceptible than carp species (5, 7). The 96 h LC50 of cypermethrin is 3.14 μg/L in rainbow trout (*Oncorhynchus mykiss*) (34) and 4.0 μg/L in Indian carp (*Labeo rohita*) (10). But deltamethrin is described to be more toxic in common carp (*Cyprinus carpio*) than in rainbow trout on the contrary (35). Acute toxicity also influences viability of embryos and leads to significant increase of dead larvae even if concentarion is orders of magnitude less (31, 36).

### *Chronic toxicity*

Chronic toxicity effects can occur at exposure levels far below the concentration that causes lethality. Sublethal biological responses include behavior changes, reduced growth, immune system effects, endocrine effects including decrease of reproductive success, histopathological and biochemical changes (12). Disturbance of the non-specific immune system is connected with decreased production of leucocytes. Changes of colours and integrity of body surface develop during the weeks of exposure (37). Early life stages are more susceptible to chronic toxicity of pyrethroids than adult fish (5, 12, 38). Fingerlings of Indian carp change shape of their bodies in sublethal exposure. They become lean towards the abdomen position compared to the control fish and they seem to be under stress, but this is not fatal (10).

## Toxicokinetics

Fish in general are exposed to pyrethroids through their gills, which are multifunctional and complex organs with which fish make intimate contact with their ambient water (39). Py-

rethroids are attracted to the non-water soluble components of cells due to their lipophilicity and permeate through the gills easily, even from water containing low levels of pyrethroids. This is a contributing factor in the sensitivity of the fish to aqueous pyrethroid exposures (40, 41).

When rainbow trout body was studied, the greatest amount of radiolabeled fenvalerate residues were found in the bile, then in the fat deposits and followed by the liver, gill, kidney and red blood cells. Concentration in the brain was lower than in most other tissues (42).

Common way of detoxification is hydrolysis in liver and plasma of animals. The acid and alcohol components of pyrethroids that result from ester hydrolysis are of minimal toxicity to any animals (1, 4). Hydrolysis is followed by hydroxylation and conjugation to glucuronides and sulphates, which are excreted in urine (4). But fish treated by pyrethroids do not show significant levels of ester hydrolysis products in urine or bile. It seems that permethrin elimination from fish is quantitatively different from that reported in mammals and birds, with oxidative degradation predominating and ester hydrolysis constituting a minor reaction (7). Oxidation products are most common, primarily due to ring hydroxylation and side chain oxidation reactions in fish (1, 7). Because of lack of hydrolysis detoxification, products of ester hydrolysis are rarely found (1) and only low levels could be confirmed (7).

Toxicokinetic experiments indicate that fenvalerate elimination rate in rainbow trout is much slower than in birds and mammals (1). The half-lives for elimination of several pyrethroids by trout are all greater than 48 h, while half-lives of elimination in birds and mammals range from 6 to 12 h (7).

## Toxicodynamics

Pyrethroids bind to a receptors at the sodium gate of neuron and prevent it from closing fully. The resulting steady leakage of sodium ions into the neuron creates a less stable resting state and the neuron is susceptible to repetitive firing of nerve, which leads to hyperactivity, tremors and tetany (43, 44).

Effect of pyrethroids in mammals and insects depends on stereospecificity highly. Some isomers demonstrate strong potency and their mirror image isomers show almost no toxicity. The available data for fish are not so uniform (1, 7). Fish seems to be equally sensitive to both cis and trans isomers of permethrin (1). In contrast stereospecific influence of fenvalerate toxicity on fish is similar to that of mammals. The 2S pair of isomers is 3.3 times more toxic to fathead minnow (*Pimephales promelas*) than technical mixture with all four isomers (1, 45). Recent research indicates stereoselectivity in the estrogenic activity of permethrin, which results from stereoselective biotransformation of the parent compound to more estrogenic metabolites. 1S-cis-permethrin has a higher activity than the 1R-cis enantiomer (46).

Synthetic pyrethroids have deleterious influence on Ca-ATPases and other ATPases in vertebrates and invertebrates so additional toxic effect must be considered (1). Fish treated by cypermethrin show inhibition of gill Na+/K+ -ATPase activity which induce osmotic imbalance and influence maintenance of osmotic and ionic homeostasis (11).

It is difficult to differentiate between type I and type II syndromes in fish. Both types of pyrethroids cause similar neurological symptoms and fish generally become inactive before death (7).

## Clinical symptoms of poisoning

The following clinical symptoms are observed during acute toxicity tests on rainbow trout and common carp: accelerated respiration, loss of movement coordination, fish lay down at their flank and move in this position. Subsequent short excitation stage (convulsions, jumps above the water surface, movement in circles) changes into a resting stage, and another short excitation period follows again. In the end fish fall into damp, move mainly at their flank. Respiration is slowed down, the damp phase and subsequent agony are very long (34, 47). Similar neurological symptoms could be observed after 2 weeks of exposure to subacute concentration of deltamethrin (1.46 µg/L) on monosex Nile Tilapia (*Oreochromis niloticus*). It is accompanied by colour darkening of the body surface, slight erosions and/or rotting of fins and tail, slimness, general loss of fish scales, eye cataract and sometimes exophthalmia. Internally, there is general congestion of the liver, kidneys, gills and blood in the abdominal cavity (37). Loss of equilibrium, vertically hanging, gill flailing, erratic swimming, swimming at the water surface, air gulping from the water surface or staying mo-

tionless on the aquarium bottom are observed during tests of acute toxicity of deltamethrin on the fry rainbow trout. The toxicity and presence of symptoms depends on increasing concentration and exposure time. Colour darkening is observed at concentrations higher than 8 µg/L (48). Study of acute cypermethrin toxicity on rainbow trout describes the almost identical neurological symptoms again (gill flailing, hyperactivity, loss of buoyancy and inability to remain upright) (27) and on common carp abnormalities of movement again and hyperactivity are described especially (49). Necropsy after acute toxicity tests on rainbow trout and common carp reveales watery mucus on body surface, excess fluids in body cavity and congestion of visceral vessels (2). Acute toxicity of cypermethrin in silver catfish (*Rhamdia quelen*) causes loss of equilibrium, vertical hanging in water, rapid gill movement, erratic swimming, sudden swimming motion in a spiral fashion after long periods of inactivity and sudden movement after prolonged inactivity in the tank bottom (50). Respiration and movement abnormalities are described mainly (30, 51).

## Endocrine and reproductive disruption

Cypermethrin reduces the fertilization success in atlantic salmon (*Salmo salar*). It inhibites ability of male salmon parr to detect and respond to the female salmon priming pheromone PGF2α. The increase in expressible milt and the levels of plasma sex hormones are reduced in the presence of the pyrethroid as the result of impaired olfactory detection of the priming pheromone (32).

## Biochemical and haematological profiles

Reduction in hepatic glycogen accompanied by increased level of plasma glucose is a common reaction of fish against xenobiotic insult followed by metabolic stress (51-54).

In rainbow trout cypermethrin causes significantly decreased concentration of ALP and significantly increased concentration of ammonia, AST, LDH, CK and lactate in blood plasma (34). In common carp bifenthrin causes increased concentration of ammonia, AST and CK too (54). In silver catfish cypermethrin causes increasing of levels $Na^+$, $K^+$, $Mg^{2+}$, P, urea, glucose, cholesterol, creatinine, AST and ALP, whereas total protein, triglyceride and ALT levels are reduced (50). In common carp deltamethrin causes decreased concentration of total protein in blood plasma (47).

An increase of plasma ammonia level is supposed due to an increase of amino acids catabolism and due to an inability to convert the toxic ammonia to less harmful substances and failure of ammonia excretion. Decrease of the levels of free amino acids accompanied by increase of the activities of AST, ALT and GDH in the vital organs is seen, because the amino acid catabolism is one of the main mechanisms, which ensure immediate energy demand to the fish (55). An increase of AST and CK indicates tissue impairment based on the stress (56). The increase of LDH level is connected with metabolic changes, i.e. the glycogen catabolism and glucose shift towards the formation of lactate in stressed fish, primarily in the muscle tissue (52). Metabolic stress induced by pyrethroids is accompanied by changes in levels of enzymes of antioxidant defense (57, 58).

Studies of haematological parameters are inconsistent. In catfish (*Heteropneustes fossilis*) deltamethrin causes a significant increase in RBC, but a small decrease in Hb, MCV, MCH and PCV (59). In common carp acute intoxication of deltamethrin causes decrease in RBC, Hb and PCV and has no effect on MCV, MCH, MCHC, total leukocyte count and relative as well as absolute counts of lymphocytes, monocytes, neutrophil granulocytes and their developmental forms (47). In rainbow trout cypermethrin causes a significant increase in the levels of RBC and a significant decrease in the Hb, MCH, MCHC, thrombocyte count and erythrocyte sedimentation rate (60). But only significant decrease in count of developmental forms of myeloid sequence and the segmented neutrophilic granulocytes is described in another acute toxicity test with cypermethrin and any effect on the haematological indicators such as RBC, Hb, PCV, MCV, MCHC, MCH and leukocytes (34). Elevation of the relative and absolute monocyte counts is described in common carp treated by bifenthrin (54). Deltamethrin causes decreased lymphocyte and basophile percentages and decrease of total leukocyte and erythrocyte counts, Hb and PCV simultaneously with serious hypoproteinaemia, hypoalbuminaemia, hypercholesterolaemia, hyperglycaemia in Nile tilapia exposed to subacute concentration for weeks (37).

## Post-mortem findings

Severe teleangioectasiae are revealed in secondary lamellae of gills, with the rupture of pillar cells in 50% of fish treated by bifenthrin (54). The most common gill changes of fish treated by deltamethrin are desquamation and necrosis. It is followed by the lifting of the lamellar epithelium, oedema, aneurism, hyperplasia of epithelial cells and fusion of the secondary lamellae. These changes are results of direct responses of gill to the action of deltamethrin and simultaneously defense responses of organism against toxicant to make it more difficult to access to blood stream (61).

Bifenthrin causes degeneration of hepatocytes, especially in periportal zones, in 40% of treated fish. Affected hepatocytes show pycnotic nuclei and many small or single large vacuoles in the cytoplasm. Vacuole shape is typical for fatty degeneration of liver. It can imply the influence of pyrethroids in the digestive tract. (54).

Deltamethrin destructive effects in fish kidney are characterized by degeneration in the epithelial cells of renal tubules, pycnotic nuclei in the haematopoetic tissue, dilatation of glomerular capillaries, degeneration of glomeruli, intracytoplasmatic vacuoles in epithelial cells of renal tubules with hypertrophied cells and narrowing of the tubular lumen (61).

## Factors influencing pyrethroids toxicity

A lot of factors can modulate the toxicity. Many synthetic pyrethroids have their 96 h LC50 values under 1µg/L, while chronic toxicity can be recorded at one to two orders of magnitude lower than that (5). Fish toxicity studies vary widely in their methodology (e.g., static conditions vs. flow-through exposures, nominal concentrations added to the water vs. measured concentrations). A lot of studies in standardized water demonstrate extraordinary toxicity, however field trials show the pyrethroids to be less potent than expected from laboratory studies. It is determined that pyrethroids, with their extremely low water solubility and high affinity for particulate matter in solution, do not remain bioavailable for uptake by the fish in the field ponds. When the pyrethroids molecules bind to the suspended solids or the sediment, the resultant toxicity is orders of magnitude less than predicted by the clean water assays (1).

Currently available formulations of pyrethroids are oil based, emulsifiable concentrates (EC). The emulsifiable formulation keeps the pyrethroids in solution longer compared to the technical chemicals and the pyrethroids adsorb to the glass quickly. Pyrethroids tend to bind to the glass and plastic (62). EC formulations are usually two to nine times more toxic than the technical grade forms, most likely due to synergistic interactions (63).

The ionic characteristics of the water can exert influence on the toxicity of pyrethroids to fish. Water hardness (summary $Ca^{2+}$ + $Mg^{2+}$) is shown to be a factor in bluegill (*Lepomis macrochirus*) susceptibility to fenvalerate. The LC50 values are twofold higher in very soft water, compared to hard water. Increased toxicity on bluegill fry is recorded when salinity raises (64). Pyrethroids are more toxic at lower temperatures and conversely fish are more susceptible at lower temperatures (1, 5, 13, 44). There is a possible increase in the toxic impact of pyrethroids on reproduction during spawning season in the cold water (32).

Pyrethroids appear to be generally more toxic to smaller fish than larger ones (5, 13, 51). Fish embryos appear to be less sensitive to pyrethroids than larvae (12).

Toxicity of pyrethroids is dramatically influenced by the presence of particulate matter in the water column, probably through adsorbtion of the very lipophilic toxicant molecules to the suspended matter, sediment and dissolved organic matter (40, 65). That is why adsorbtion of pyrethroids is more quick in system like farm ponds with organic matter than in typical streams (12).

Piperonyl butoxide is commonly added to pyrethroid products to enhance the toxic effects of the active ingredient. Piperonyl butoxide inhibites a group of enzymes, which are involved in pyrethroid detoxification (12).

## Conclusion

Pyrethroids are predominant class of insecticides. Their widespread use represents an increasing threat of water pollution. Investigation of their properties in connection with environment, acute and chronic effects and potential bioaccumulation must continue thoroughly. Research on non target species including fish should be really detailed.

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## VPLIV PIRETROIDOV NA RIBE

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**Povzetek:** Piretroidi spadajo med najbolj pogosto uporabljene pesticide po vsem svetu. Njihova masovna uporaba ogroža naravno okolje, vključno z vodnimi ekosistemi. Čeprav se piretroidi v tleh in rastlinah hitro razgradijo, so za ribe zelo strupeni. Glede na svoje značilnosti se piretroidi delijo v dve skupini, tip I in II. Oba povzročata podobne nevrološke simptome. Piretroidi vplivajo na delovanje natrijevih kanalčkov v živčnih celicah, piretroidi tipa II poleg tega vplivajo tudi na kloridne in kalcijeve kanalčke. Ključnega pomena pri zastrupitvi rib s piretroidi je njihovo počasnejše izločanje kot pri pticah in sesalcih. Piretroidi se hitro absorbirajo preko škrg, po krvi pridejo v žolč, jetra, ledvice in rdeče krvne celice, kjer se presnavljajo s hidrolizo, hidroksilacijo in vezavo na glukuronide in sulfate. Akutna zastrupitev rib se kaže z motnjami v gibanju in dihanju ter smrtjo. Kronična izpostavljenost piretroidom pri ribah povzroči spremembe v obnašanju, krvni sliki, histopatološke spremembe, zmanjšano rast ter vpliva na imunski in endokrini sistem. V obeh primerih pa je tudi prizadeta reprodukcijska sposobnost. Toksičnost piretroidov je odvisna od številnih notranjih in zunanjih dejavnikov.

**Ključne besede:** piretroidi; nevrotoksičnost; občutljivost; fiziološke motnje

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**Original Scientific Article**

# THE PRESERVATION OF TRICLABENDAZOLE IN BAITS FOR FREE-LIVING RED DEER (*CERVUS ELAPHUS* L.) DURING THE PRE-CONSUMMATION PERIOD

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**Summary:** Triclabendazole is anthelmintic drug that is among other successfully used to control large American liver fluke (*Fascioloides* magna) infections in several deer species. In attempts to control deer fascioloidosis, medicated corn was the main route of triclabendazole administration. However, despite the fact that medicated feed offers several advantages for treatment of wild animals, its efficacy largely depends on the amount of active ingredient that may be influenced by prolonged pre-consummation period, rainfalls, freezing, handling, etc. In this study selected mixture (according to handling characteristics and attractiveness to deer species) and corn alone were treated with Fasinex® 10%, exposed to environmental conditions and analyzed with high performance liquid chromatography after 1, 3 and 5 days (expected time of consummation). Both baits proved to be an excellent triclabendazole carrier containing on average 31.76 g/kg of triclabendazole per kg of mixture and 30.18 g of triclabendazole per kg of corn after five days of pre-consummation period. However, majority of triclabendazole in medicated corn was kept on its surface, thusly being highly sensitive to environmental conditions.

**Key words:** red deer; triclabendazole; stability; pre-consummation period; *Fascioloides* magna

## Introduction

Triclabendazole is anthelmintic drug that binds to the parasite's tubulin, consequently impairing the normal cellular mechanisms particularly of the trematodes of the genera *Fasciola*, *Fascioloides* and *Paragonimus* (1, 2). It is having a unique broad spectrum of activity against immature, young and adult trematodes (3, 4). Wildlife veterinarians were especially attracted to triclabendazole following its observed efficacy against naturally occurring *Fascioloides magna* infections (4).

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Fascioloidosis is a parasitic disease of special interest in Europe, as *F. magna* is an autochthonous member of the North American parasite fauna where its main hosts are white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*Odocoileus hemionus*) and wapiti (*Cervus elaphus nelsoni*). On the other hand, in Europe, it represent invasive, non-native parasite that cause severe disease in red (*Cervus elaphus*), fallow (*Dama dama*) and roe deer (*Capreolus caplreolus*) and mouflon (*Ovis ammon musimon*). Therefore, after the *F. magna* infection was detected in red deer from Croatia, causing increased mortality, reduced reproductive success, decreased body weight and trophy value (5), triclabendazole was chosen for treatment of free-ranging populations in order to

control disease and prevent their further spreading (6,7). Especially after the mouflon, fallow deer and roe deer population in several enclosed hunting grounds in Croatia were depleted by *F. magna* (unpublished data).

The use of medicated feed (baiting strategy) is particularly important in the management of wildlife diseases, as it enables group treatment and does not require immobilization or any kind of handling. However, in the case of low consumption rate, treatment efficacy is among other, influenced by the preservation of the active component during the pre-consummation period. Therefore, the most attractive bait which contains a sufficient amount of drug for a longer time period should be offered to the animals. Especially in the cases where rough or muddy terrain does not allow regular visits of wildlife managers and veterinarians to that particular area. Of course, all new baits should be processed previously through an adaptation period i.e. by exposing of non-medicated baits.

Since fascioloidosis is the most important parasitic disease of red deer in Eastern Croatia, in this study we tried to find out the most acceptable medicated bait that will contain sufficient amount of active ingredient even after prolonged pre-consummation period and less careful handling.

## Material and methods

### *Study area and environmental conditions*

The experiment was performed in the open state hunting ground No. XIV/9 "Podunavlje-Podravlje" in north-eastern Croatia (Baranja region). After preparation, tested mixtures and medicated corn were kept at room temperature (18-22°C) for the first 24 hours. Daily temperatures for the study period were obtained from three meteorological stations closest to the research area; Osijek, Brestovac-Belje and Beli Manastir. For each day the average temperature was calculated from three measurement points, at 7 a.m., 2 p.m. and 9 p.m. The average daily temperature during March for the Baranja region was 8.5°C±2.3 (meteorological station Osijek), 8.4°C±2.2 (Brestovac-Belje), and 8.5°C±2.4 (Beli Manastir). There were 13 rainy days and 1 day with snow/rain (station Osijek), and 11 days with rain for the Brestovac-Belje station and the Beli Manastir station.

### *Baits*

In this trial nine different mixtures as carriers of triclabendazole were prepared and analyzed for their handling characteristics, viscosity, attractiveness to red deer, consumability and stability of the mixture in natural conditions. Based on these characteristics mixture containing corn grits, vegetable oil, vegetable fat, chalk powder, aromatic powder and salt was chosen for the second step of the analysis. In brief, mixture ingredients were chosen as carriers (corn grits, chalk powder), shaper and evaporation retardant (vegetable fat and oil), attractants (aromatic powder and salt) and regulator of consumption rate (salt). In the second step or the analysis, triclabendazole was added to selected mixture and to corn alone in a way that approximately 210 to 240 g of such applied bait contained a sufficient amount of triclabendazole for single-dose treatment of 100 kg red deer (suggested dose is 60 mg/kg of body weight) (4). Following inspection of their physical characteristics, tested baits were placed in a feeding station (with no ground contact and protected from the rain). Bait mixture was shaped in ball-like objects of, approximately, desired weight. Corn alone was mixed with Fasinex® 10% (500g of corn + 120 ml of Fasinex®), and left to dry for one day. The third day after preparation, sample Ac (corn) was divided in two parts; Ac1 was washed out and submitted for HPLC analysis. At the same time, remaining part, sample Ac2 was submitted without the washing procedure. Sample Bc was submitted on the 5$^{th}$ day after preparation. Baits were exposed to environmental conditions during March, since that month was chosen for treatment of the wild red deer population.

### *HPLC procedure*

Triclabendazole standards were prepared by diluting 10% Fasinex® (mass concentration of triclabendazole was 100 g/L) with methanol to reach final concentrations (20, 24, 28, 32, 36 and 40 g/kg) and a volume of 10 mL. In the next step a 10 mL of diluted Fasinex® with a known amount of triclabendazole was added to 100 mg of corn grits, mixed on a low speed shaker for 15 minutes and centrifuged at 3500 x g for 10 minutes. Standards were freshly prepared each day. 10 ml of methanol was added to 100 mg of each analyzed sample,

mixed on a low speed shaker for 15 minutes and centrifuged at 3500 x g for 10 minutes. A total of 1 ml of supernatant was put in vials, and 10 µL of supernatant was analyzed by high performance liquid chromatography (HPLC). Each sample was analyzed three times and the concentration was obtained from the calibration diagram.

## Results

Following the test of consistency, color and smell after the initial 7 days of exposure (without triclabendazole), selected mixture was best consumed when offered to free-ranging red deer (90% of baits were consumed within three days) and therefore was chosen for further analysis. The second step was to evaluate the amount of triclabendazole in selected baits (mixture and corn) during the expected time of consummation (up to 5 days). The results of the HPLC analysis are summarized in the Table 1. The HPLC analysis revealed that triclabendazole concentrations in corn 3 days after treatment with Fasinex® 10% are at 24.43 g per 1 kg of prepared feed (average value calculated from three repeated measurements). On the other hand analysis of the washed corn indicated that the majority of drug was on the surface of the corn (on average only 1.28 g of triclabendazole per kg of prepared corn entered inside it). Following prolonged exposure of 5 days (starting from the day of preparation) medicated, non-washed corn contained on average 30.18 g of triclabendazole/kg. Analyzed mixture, divided as samples A, B and C, contained on average 27 g of triclabendazole per kg of mixture (after 1 day of storage), 33.78 g/kg (after 3 days) and 31.76 g/kg (after 5 days).

**Table 1:** Triclabendazole content in baits on 1st, 3rd and 5th day. Samples A, B, C represent mixture No 9. Samples Ac1, Ac2 and Bc represent medicated corn (Ac1 – washed corn)

| | Mixture No 9 | | | Corn | | |
|---|---|---|---|---|---|---|
| 0 | Sample A | Sample B | Sample C | Sample Ac1 | Sample Ac2 | Sample Bc |
| 1 | 27 g/kg | | | | | |
| 3 | | 33.78 g/kg | | 1.28 g/kg | 24.43 g/kg | |
| 5 | | | 31.76 g/kg | | | 30.18 g/kg |

## Discussion

The potential of corn as a drug carrier for treatment of free-ranging red deer against *F. magna* and other trematodes is known from previous studies (8,9,10). However, in mentioned studies there were no data on distribution and preservation of triclabendazole in baits (medicated feed) during the pre-consummation period. In our study, results of the HPLC analysis, concentrated on the expected consummation period, revealed that triclabendazole concentrations in corn 5 days after treatment with Fasinex® 10% are at suggested therapeutic level. According to previous studies suggested dose for wapiti (closest examined relative of red deer) is 60 mg/kg of body weight (4), while even dose of 100 mg/kg shows no toxic effect. Analysis of the washed corn indicated that the majority of active ingredients remained on the surface of the corn. Following 5 days of exposure (starting from the day of preparation) observed slight increase in triclabendazole concentration in medicated, non-washed corn is attributable to evaporation and the consequent increase in dry matter of the analyzed sample.

Pre-consummation period is expected time between exposure of medicated feed and their consummation by targeted species. In the case when large numbers of targeted animals visits feeding stations daily, especially when combined with low feed availability pre-consummation period can be significantly reduced. In example, during the treatment of white-tailed deer Qureshi et al. (8) exposed medicated corn for a period of 1wk. According to them, the average efficacy of triclabendazole in treatment of trematode *F. magna*, administered via corn during the three year period, was 63%. We assume that the majority of medicated corn in that study was taken by deer during the first few days, period when triclabendazole concentrations were at the highest level. Therefore, the best therapeutic results could be achieved within five days, as shown in this study. Similar and even better results were obtained by Ursprung et al. (10) in the period between 2000 and 2005.

To summarize, corn as the sole drug carrier has obvious advantages such as easy preparation of medicated feed, easy distribution and field evidence from previous studies that most probably deer will not eat large quantities of concentrated feed (by that preventing the overdosing). On the other hand, the disadvantages are in the fact that the majority of triclabendazole are kept on the corn surface and are exposed to all environmental factors (especially rain and low temperatures), as well as to mechanical insults that can remove drug from the corn surface. This finding suggests that handling of treated corn should be kept to a minimum and that corn must be protected from diverse atmospherilia. In the other cases, when populations are smaller and especially when natural feed resources are available (as seen in the Croatian hunting grounds) the deer will reduce their intake of supplemented feed, thereby prolonging the pre-consummation period. To minimize these negative effects, March was chosen for treatment of wild red deer. It is expected that in that time red deer metabolism should already be shifted from catabolism to anabolism (11), requiring increased food intake while natural high quality feed resources are still rather scarce, forcing the red deer to consume exposed baits.

In the case of analyzed mixture, the 5 day trial period proved the sufficient preservation of the triclabendazole, which was more or less evenly distributed throughout the mixture. Such triclabendazole distribution minimizes the effect of external agents and enhances preservation of the active ingredient. Furthermore, rather high ratio of salt in the mixture (approximately 5%) prevents the overdosing and minimizes negative effects of low environmental temperatures.

Our results indicate that both, corn alone and analyzed mixture are suitable carriers for triclabendazole. We can conclude that triclabendazole concentrations on medicated corn are at suggested dose, required to treat deer against large American liver fluke for at least five days. However, if treated carefully and without rainfall (to avoid mechanical removal and washing out effect). In the case of smaller deer populations (expected longer period of consummation) and rainy periods, analyzed mixture proved to be a successful triclabendazole carrier and potential tool in *F. magna* strategy controls, after an initial period of habituation.

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## OHRANJANJE TRIKLABENDAZOLA V VABAH ZA PROSTO ŽIVEČO JELENJAD *(CERVUS ELAPHUS* L.*)* V PREDKONZUMACIJEM OBDOBJU

Z. Janicki, A. Slavica, K. Severin, D. Konjević

**Povzetek:** Triklabendazol je antiparazitik, ki se uspešno uporablja pri zatiranju ameriškega velikega metljaja *(Fascioloides magna)* pri več vrstah jelenov. Za zatiranje metljavosti pri jelenih se v glavnem uporablja krma s triklabendazolom. Krma z vsebnostjo zdravil ponuja številne prednosti za zdravljenje divjih živali, njena učinkovitost pa je odvisna od količine aktivne sestavine, na katero vpliva dolžina obdobja pred zauživanjem krme, količina padavin, temperatura, skladiščenje krme ipd. V članku smo preučili krmno mešanico za jelene in samo koruzo, ki sta bili tretirani z 10 % raztopino Fasinex®-a in izpostavljeni zunanjim vplivom. Po 1, 3 in 5 dneh (pričakovani čas konzumacije) smo analizirali vsebnost triklabendazola s tekočinsko kromatografijo visoke ločljivosti. Obe vabi sta se izkazali kot odlično sredstvo za prenos triklabendazola; v povprečju je bila vsebnost zdravila 31,76 g triklabendazola na kg krmne mešanice in 30,18 g triklabendazola na kg koruze po petih dneh. Vendar je na koruzi večina triklabendazola ostala na površini, zaradi česar je bolj občutljiv na vplive okolja.

**Ključne besede:** jelen; triklabendazol; stabilnost; obdobje pred zaužitjem; *Fascioloides magna*

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**Original Scientific Article**

# BOVINE TUBERCULOSIS IN CATTLE DURING THE IMPLEMENTATION OF OFFICIAL CONTROL MEASURES IN REPUBLIC OF MACEDONIA FOR THE PERIOD 2007-2009

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**Summary:** Tuberculosis in cattle has been subject of different control programmes since late 40's of the last century. Latest *Multi-Annual National Programme for Eradication of Bovine Tuberculosis in Cattle in Republic of Macedonia* has been adopted and started implementing in 2007. This study is an evaluation of the results from implementation of this program.
A retrospective and descriptive study has been carried out. Demographic and epidemiological characteristics of the bovine tuberculosis were evaluated. An average of 160 784 (61.96%) of total number of cattle were covered by this programme. The single tuberculin test (STT) was positive in 1 021 (0.63%) of the tested animals. Only 952 (93.21%) animals that reacted positive to the single tuberculin test were subjected to comparative tuberculin testing, and an average of 390 (40.95%) of them were declared as positive. In 2007, 43 573 herds (holdings) were tested for bovine tuberculosis, where 173 were found to be positive; in 2008 from 43 753 tested herds, 253 were positive and in 2009 from 42 714 tested, 265 were positive. All animals found positive for bovine tuberculosis were slaughtered in the sanitary slaughterhouse and the milk was declared as not fit for human consumption.
Regarding bovine tuberculosis, Republic of Macedonia is considered as country with low prevalence with overall average prevalence of 0.002% for the period 2007-2009. The North-west, South-west and Eastern regions of the country were more intensively affected by the disease. For more efficient control, the number of tested cattle should be increased, followed by increasing of the sanitary measures and epidemiological tracing–back.

**Key words:** tuberculosis; cattle; tuberculin test; zoonosis; food safety

## Introduction

Bovine tuberculosis (bovine TB) is a chronic bacterial disease of animals and humans caused *by M. bovis and M. caprae*. Large number of countries are reporting bovine TB as a major infectious disease among cattle, other domestic animals and certain wild animals. Transmission to humans is major public health issue [1]. Bovine TB causes severe economic losses in livestock due to loss of production, mortality, and condemnation of carcasses [2].

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In 1882, the time of Koch's discovery of the etiological agent, between 20% and 40% of the cattle in many European countries had tuberculosis [3].

Developed countries, control bovine TB by testing and slaughtering the animals, compulsory pasteurization of milk and minimizing the risks for human infections. In those countries, clinical cases of tuberculosis in cattle are seldom occurring due to the programmes for control and eradication, which enables early detection and presumptive diagnosis with consecutive elimination of infected animals before appearance of clinical signs [4]. Control programmes in western European countries were established as far as the 1930-s [5], and in central European countries in

the 1960-s [6]. Sweden declared it's officially tuberculosis free status in 1958 [7], USA in 1991 even though the eradication campaign started in 1917 [8]. In Canada the disease was brought under the mantle of official notification and eradication program in 1923. All Canadian cattle herds that were not under quarantine were recognized as being TB-free in 1997 [9].

Bovine TB is present in the Balkan region. According to the OIE, the average prevalence in Albania for the period 2007-2008 was 0.018%, in Serbia for the period 2007-2009 was 0.006%, and in Greece for the same period reported average prevalence of 0.052% [10]. The results are provided in Table 1.

**Table 1:** Cattle population and bovine TB in four Balkan countries according to OIE *

| | Cattle population (No. of) | | | Bovine TB (No. of) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Year | Country | Anim. | Establish. | New outbr. | Susceptib. Anim. | Cases | Deaths | Destr. | Slaugh |
| 2007 | Albania | 634 000 | 256 356 | 18 | 71 037 | 113 | 0 | 0 | 113 |
| | Bulgaria | 582 594 | 199 610 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Greece | 880 110 | 32 674 | 42 | 2 921 | 328 | 17 | 0 | 1 664 |
| | Serbia | 1 735 248 | 299 264 | 36 | 256 | 63 | 0 | 0 | 7 |
| 2008 | Albania | 699 502 | 271 230 | 15 | 35 332 | 137 | 0 | 0 | 97 |
| | Bulgaria | 584 468 | 195 071 | 2 | 2 | 2 | 0 | 0 | 1 |
| | Greece | 893 046 | 35 250 | 38 | 2 835 | 438 | 0 | nr | 121 |
| | Serbia | 1 057 000 | 305 647 | 35 | 764 | 94 | 0 | 1 | 58 |
| 2009 | Albania | 541 | 226 443 | 7 | 2 890 | 54 | 0 | 0 | 54 |
| | Bulgaria | 584 283 | 127 060 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Greece | 749 623 | 38 041 | 48 | 3 824 | 525 | 3 | 0 | 222 |
| | Serbia | 1 087 504 | 21 5226 | 32 | 857 | 73 | 0 | 0 | 54 |

nr = not reported; anim. = animals; outbr=outbreaks; suscept=susceptible; destr=destroyed;slaugh.=slaughtered
*There are no data for Kosovo and Montenegro

Bovine TB infection in cattle is usually diagnosed in live animals on the basis of delayed hypersensitivity reactions. The World Organization for Animal Health (OIE) prescribes the tuberculin test (TT) as a reliable method for screening of bovine TB [1]. It involves measuring skin thickness before and after tuberculin is injected into the measured area. The single tuberculin test (STT) uses bovine tuberculin, while comparative tuberculin test (CTT) uses both, bovine and avian tuberculin. The second test is used mainly to differentiate between animals infected with bovine TB and those sensitized to tuberculin due to exposure to other mycobacteria or related general. The TT is used generally and depends on the prevalence of tuberculosis infection and on the level of environmental exposure to the other sensitizing organisms among animals evaluated. The reactions are interpreted on the basis of appropriate schemes [1].

The objective of this study was to analyze the epidemiological situation with bovine TB in the cattle in Macedonia for the period from 1 January 2007 to 31 December 2009 and to assess the efficiency of the bovine TB eradication programme in cattle population.

## Material and methods

### *Animals and herds*

The territory of Republic of Macedonia is divided into 84 epidemiological units which correspond to administrative and territorial division of the coun-

try. The system for identification and registration (I&R) of cattle in Republic of Macedonia regulates holdings as equivalent to herds due to the large number of small farms (herds) by average of 5.99 cattle per holding during the evaluation period. The Multi-Annual Programme for Eradication of Bovine TB requires record keeping for all cattle together, not by different categories. All bovine animals older than 6 weeks were subject to testing. STT was performed by the veterinary stations contracted by the Food and Veterinary Agency (FVA). Based on their reports, STT positive animals were submitted for CTT. The CTT was performed by the Faculty of Veterinary Medicine-Skopje.

*Skin testing*

The STT involved intra-dermal injection of 0.1 ml of 2000 International Units (IU) of bovine purified protein derivate tuberculin (B-PPD) (Veterina, Croatia) in the mid-neck of the animal. Firstly, the mid-neck section was clipped with scissors and then skin-fold thickness was measured with kutimeter (calipers). The injection of B-PPD was administered via intra-dermal route in the measured place and the skin-fold thickness was measured after 72 hours. The STT reaction was considered positive when clinical signs (diffuse or extensive edema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes) were detected or increase of 4 mm or more in skin-fold thickness. Inconclusive reaction was considered when the increase in skin-fold thickness was more than 2 mm and less than 4 mm without manifestation of aforementioned clinical signs. Animals with inconclusive or positive reactions by the STT were subjected to a CTT (re-testing) after 42 days interval as desensitization period. The test was carried out by intradermal injection of 0.1 ml of 2000 IU of B-PPD (Veterina, Croatia) in the mid-neck of the animal, by simultaneous intra-dermal injection of 0,1 of 2000 IU of avian PPD tuberculin (A-PPD) (Veterina, Croatia) at a distance of 12-15 cm from the first one. Animals with increased skin-fold thickness at the bovine site of injection higher than 4 mm than the reaction at the avian injection site were declared as infected with causal agent of bovine TB. The CTT and the interpretation of the results were performed by the Faculty of Veterinary Medicine - Skopje [1, 11].

*Reactors' management*

According to the *Multi-Annual National Programme for Eradication of Bovine Tuberculosis in Cattle in Republic of Macedonia*, all cattle found positive by CTT were removed from the herd and slaughtered in sanitary slaughterhouse within a period of maximum one month. The movement and trade of the other bovine animals from the infected herds, has been prohibited. The animal holdings were cleaned and disinfected. All farmers, whose cattle were slaughtered, received reimbursement funds by the FVA. The milk of the suspected and infected animals was declared as non-fit for human consumption. Following the *post-mortem* inspection, the meat from slaughtered cattle was used in the meat processing industry following a heat treatment ensuring destruction of the bacillus. In cases of generalized form of TB, the cattle corpses were confiscated and safely disposed [11].

## Results

In all 84 epidemiological units STT was performed on 60.9% in 2007, 69.4% in 2008 and 55.5% in 2009 from all bovine animals. The STT was positive in 0.50%, 0.70% and 0.71% of all bovine animals subjected to STT in 2007, 2008 and 2009 respectively. Out of all STT positive reactors, an average of 92.85% were re-tested with CTT (90.51% in 2007, 95.37% in 2008 and 92.67% in 2009: Table 2). In comparison to 2007, a significant increase of 39% of CTT re-tests were performed in 2008. Furthermore, there were total 30% more positive animals in 2008 in comparison to 2007. A decline of 22% in the total performed CTT re-tests was observed in 2009, with total 13% less positive animals than in 2008.

**Table 2:** Results from the 2007 to 2009 National eradication program

| | Years | | | Averages |
|---|---|---|---|---|
| | 2007 | 2008 | 2009 | 2007 to 2009 |
| Total No of cattle | 261 058 | 259 479 | 257 900 | 259 479 |
| Total No STT tested | 158 965 | 180 150 | 143 237 | 160 784 |
| % of STT tested (from all cattle) | 60.89% | 69.43% | 55.54% | 61.95% |
| Total No STT positive | 801 | 1 254 | 1 010 | 1 021 |
| % of STT positive (from STT tested) | 0.50% | 0.70% | 0.71% | 0.64% |
| Total No CTT tested | 725 | 1 196 | 936 | 952 |
| % CTT tested (from STT positive) | 90.51% | 95.37% | 92.67% | 92.85% |
| Total No CTT positive | 319 | 454 | 396 | 390 |
| %CTT positive ( from CTT tested ) | 44% | 37.96% | 42.31% | 41.42% |

During the evaluation period of this study, animals positive on bovine TB were detected in 38 epidemiological units (45.23% of all units). The disease was not confirmed in the central-west and south-east parts of the country. The presence of the disease was confirmed constantly in 20 epidemiological units in the north-west, south-west and the eastern part of the country. In 5 epidemiological units: Vinica, Gostivar, Probistip, Sveti Nikole and Struga, the disease was confirmed for the first time in 2008 and was present also in 2009. Those epidemiological units are situated in the north-west, east and the south-west part of the country. Despite the newly confirmed bovine TB cases in 2008, in 2009 the number of epidemiological units affected by the disease decreased from 19 to 13. Furthermore, in 2009 there were no newly confirmed cases of bovine TB in the epidemiological units that were declared as free from TB in the previous years (Table 3).

Geographical presentation of the presence of the bovine TB in different areas is given in fig. 1, 2 and 3, where the red triangles stand for the epidemiological units affected with the disease.

The average point prevalence of bovine TB among the tested animals in the evaluation period was 0.002% (0.0020% in 2007, 0.0025% in 2008 and 0.0027% in 2009).


**Figure 1:** Bovine tuberculosis occurrence in 2007

**Table 3:** Bovine TB positive epidemiological units, animals and herds

| epidemiological units where CTT was performed | Bovine TB in 2007 | | | Bovine TB in 2008 | | | Bovine TB in 2009 | | |
|---|---|---|---|---|---|---|---|---|---|
| | CTT animals tested | CTT Positive animals | No. of positive herds | CTT animals tested | CTT Positive animals | No. of positive herds | CTT animals tested | CTT Positive animals | No. of positive herds |
| Bitola | 43 | 9 | 5 | 79 | 7 | 5 | 48 | 9 | 7 |
| Mogila | 19 | 1 | 1 | 28 | 4 | 2 | 43 | 5 | 4 |
| Novaci | 20 | 2 | 1 | 31 | 5 | 3 | 29 | 2 | 2 |
| Veles | 7 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Vinica | 2 | 0 | 0 | 4 | 1 | 1 | 4 | 1 | 1 |
| Vrapciste | 43 | 8 | 3 | 10 | 2 | 2 | 6 | 4 | 4 |
| Gostivar | 0 | 0 | 0 | 18 | 7 | 6 | 26 | 6 | 6 |
| Delcevo | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Kamenica | 33 | 0 | 0 | 11 | 0 | 0 | 14 | 0 | 0 |
| Rosoman | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Kichevo | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| Kochani | 19 | 1 | 1 | 3 | 1 | 1 | 12 | 2 | 2 |
| Kratovo | 9 | 1 | 1 | 8 | 3 | 2 | 11 | 4 | 1 |
| Kr. Palanka | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Kumanovo | 13 | 3 | 2 | 18 | 2 | 2 | 26 | 12 | 7 |
| Lipkovo | 13 | 2 | 2 | 26 | 4 | 3 | 37 | 11 | 8 |
| Nagorichane | 21 | 9 | 4 | 19 | 4 | 2 | 18 | 4 | 3 |
| Ohrid | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 |
| Prilep | 0 | 0 | 0 | 3 | 0 | 0 | 4 | 0 | 0 |
| Probishtip | 0 | 0 | 0 | 3 | 1 | 1 | 4 | 1 | 1 |
| Konche | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Radovish | 7 | 0 | 0 | 0 | 0 | 0 | 9 | 0 | 0 |
| Resen | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Lozovo | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| Sveti Nikole | 14 | 0 | 0 | 9 | 4 | 3 | 7 | 2 | 2 |
| Ilinden | 22 | 10 | 2 | 7 | 4 | 2 | 24 | 2 | 2 |
| Petrovec | 3 | 1 | 1 | 8 | 1 | 1 | 9 | 1 | 1 |
| Gazi Baba | 3 | 1 | 1 | 4 | 2 | 2 | 9 | 3 | 2 |
| Saraj | 8 | 3 | 2 | 47 | 22 | 9 | 71 | 46 | 21 |
| Struga | 0 | 0 | 0 | 6 | 2 | 2 | 19 | 2 | 2 |
| Brvenica | 80 | 66 | 17 | 58 | 25 | 16 | 96 | 41 | 32 |
| Zelino | 76 | 52 | 43 | 327 | 143 | 86 | 147 | 68 | 53 |
| Jegunovce | 4 | 1 | 1 | 8 | 4 | 3 | 10 | 4 | 4 |
| Bogovine | 55 | 31 | 17 | 127 | 57 | 27 | 43 | 14 | 8 |
| Tearce | 22 | 9 | 7 | 68 | 31 | 23 | 34 | 22 | 17 |
| Tetovo | 180 | 108 | 61 | 248 | 115 | 47 | 164 | 129 | 74 |
| Karbinci | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| Shtip | 5 | 1 | 1 | 7 | 3 | 2 | 11 | 1 | 1 |
| Total | 725 | 319 | 173 | 1 196 | 454 | 253 | 936 | 396 | 265 |

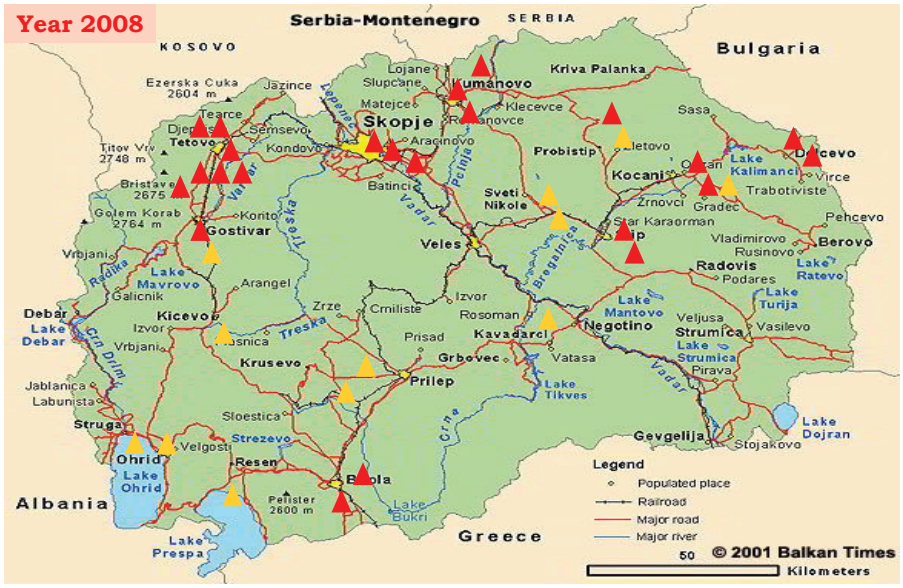


**Figure 2:** Occurrence of bovine tuberculosis in 2008. Epidemiological units first time suspected are Gostivar, Vinica, Rosoman, Kicevo, Debarca, Dolneni, Krivogastani, Probistip, Resen, Sveti Nikole, Lozovo, and Struga (yellow triangle)


**Figure 3:** Bovine tuberculosis occurrence in 2009

## Discussion

Since 1940-s, many different strategies and programmes for control and eradication of bovine TB have been carried out in Republic of Macedonia. The latest *Multi-Annual Program for Eradication of Bovine Tuberculosis* was adopted in 2007. Before the starting of this program, during the period of 2004-2006 STT was performed on 39.2% of the cattle population with prevalence of 0.045% [12]. According to our results for the first three years (2007-2009) of the implementation of the latest programme, the percentage of STT performance increased up to 61.95% with average prevalence of 0.002% of the cattle population.

For the evaluation period, out of total 84 epidemiological units, TB positive reactors were found in 38 (45.23%), where 20 (23.81%) of them were continuously giving positive reactors.

In 2007, 173 (0.4%) holdings out of 43 573 were positive in 20 epidemiological units. In 2008, 253 (0.58%) holdings out of total number of 43 753 were positive where 240 (94.86%) of the positive holdings originated from 20 continuously infected epidemiological units. In 2009, out of 42 714 total holdings, 265 (0.62%) were positive, with 253 (95.47%) originating from 20 continuously infected epidemiological units.

If we analyze the distribution of individual positive animals, all 319 positive reactors were from 20 continuously infected epidemiological units in 2007. In 2008, 439 (96.7%) out of 454 positive reactors were detected in the same 20 continuously infected epidemiological units. In 2009, 384 (97.7%) out of 393 positive reactors originated from the same 20 continuously infected epidemiological units.

The results of this study show lower prevalence of TB than the results given by various authors from other world regions: Arab peninsula (0.12%) [13]; Africa (from 0.55% to 2.1%) [14, 15, 16]; Ecuador (3.85%) and Uruguay (0.5%) [2, 17].

By analysis of the comparison of the reports submitted to OIE, it can be noted that the average prevalence of bovine TB in the neighboring countries for the same period (2007-2009) is similar (Albania 0.018%, Serbia 0.006% and Greece 0.052%). Bulgaria has reported only 2 cases of bovine TB in 2008 (0.0003%) while there are no reports from Montenegro and Kosovo [10]. The missing data from Kosovo is particularly important having in mind bordering North-West region of Macedonia with highest prevalence of TB (Table 3. the epidemiological units of Brvenica, Zelino, Tearce, Jegunovce, Bogovinje and Tetovo). *Ameni and al.* detected the lack of quarantine and smuggling of live animals across borders, as factors that promote transmission of *M. bovis* from one country to another, as well as persistent infections in such regions [18].

With exception of Greece with 23.9 animals per holding, average holding (herd) size in rest of the neighboring countries is similar to Macedonian 5.9 (Albania 2.5, Bulgaria 3.5 and Serbia 4.7) [10]. *Perez et al.* suggest that herd size play important role in spreading of TB where large farms are at higher risk than small farms [2]. Our data indicate the opposite, finding positive reactors mostly in the small holdings. This can be supported by average 1.84; 1.79 and 1.48 positive animals per infected holding for 2007/08/09 respectively.

In this study STT positive reactors were found in 0.64% of the total tested cattle, while the CTT confirmed only 41.4% of positive STT. In a survey conducted in a country with higher prevalence of the bovine BTB, a total of 4.24% of the cattle were positive to the STT and 88.6% of them were positive to the CTT [2].

The CTT was not performed in approximately 7% of the STT positive and inconclusive animals. Those animals were dead, slaughtered or sold. In other studies, awareness of the breeders for the disease was detected as a crucial factor for the eradication of the disease. The level of disease awareness among famers was related to the prevalence of the disease, the higher the prevalence of the disease is, the higher is the awareness [19, 20]. This high percentage of animals escaping the retesting with CTT has been identified as a problem where the competent authority should focus its attention to stricter implementation of the movement control measures in STT positive holdings.

## Conclusions

A period of three years (2007-2009) is insufficient for full evaluation of the efficiency and effectiveness of the *Multi-Annual National Programme for Eradication of Bovine Tuberculosis in Cattle in Republic of Macedonia.* Nevertheless it is providing some preliminary data that can be used as direction of further improvement and adjustment of the programme. By means of the implementation of the program in the first three years, the total number of tested animals has been increased giving better information for the prevalence of bovine TB in the country, distribution of infected holdings and their grouping in epidemiological units.

In general, Republic of Macedonia can be considered as a low prevalence country for bovine TB in cattle. The North-West region of the country has the highest prevalence of bovine TB. The major reasons for persistence of bovine TB in this area are insufficient movement control, incomplete depopulation of infected herds and deficient disinfection. In this part of the country, cooperation with cattle owners is also critical. For the successful implementation of TB control programme, public awareness on the importance of tuberculosis as an animal and public health risk should be raised on a higher level, particularly for the cattle farmers.

In Republic of Macedonia, bovine TB is present dominantly in small holdings, grouped in traditional areas of 20 epidemiological units. The main reasons for persistence of the disease in those holdings are weak movement control, lack of biosafety measures and joint pasture. The eradication programme should be extended also to the wild animals on the whole territory of the Republic of Macedonia, especially along the borders with Albania, Kosovo and Serbia.

The lack of data on the situation with Bovine TB in Kosovo and Montenegro must be overcome by bilateral data exchange between the veterinary authorities of those countries or by providing their reports through OIE.

The eradication programme for bovine TB should engage other diagnostic methods than TT. Molecular biology tests such as PCR as well as bacteriology must accompany STT and CTT. Low percentage of confirmation between STT and CTT (41.4%) as well as many inconclusive cases (e.g. generalized TB, cross-reactive animals etc.) can be resolved by those methods.

Government should provide stabile financing of the Program for control and eradication of tuberculosis, particularly for the timely reimbursement to the farmers and fast removal of the positive reactors from the holdings as well as for the implementation of the measures prescribed by the programme.

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## TUBERKULOZA PRI GOVEDU V ČASU UVAJANJA URADNIH UKREPOV NADZORA V REPUBLIKI MAKEDONIJI OD LETA 2007 DO 2009

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**Povzetek:** Tuberkuloza pri govedu je bila predmet različnih programov nadzora od konca 40-ih let prejšnjega stoletja. Zadnji večletni nacionalni program za izkoreninjenje tuberkuloze pri govedu v Republiki Makedoniji je bil sprejet 2007 in se je istega leta začel izvajati. Članek povzema raziskavo vrednotenja rezultatov izvajanja tega programa. Izvedeni sta bili retrospektivna in opisna študija. Ocenjene so bile demografske in epidemiološke značilnosti tuberkuloze pri govedu. V programu je bilo zajetih 160784 ali 61,96 % vseh govedi. Enotni tuberkulinski test (STT) je bil pozitiven v 1021 primerih ali pri 0,63 % testiranih živali. Le 952 živali (93,21 %), ki so se odzvale pozitivno na enotni tuberkulinski test, je bilo vključenih v primerjalno tuberkulinsko testiranje, od tega jih je bilo 390 (40,95 %) pozitivnih. Leta 2007 je bilo na govejo tuberkulozo testiranih 43573 čred (gospodarstev), kjer je bilo odkrito 173 pozitivnih, v letu 2008 je bilo od 43753 testiranih čred 253 pozitivnih in v letu 2009 od 42714 testiranih 265 pozitivnih. Vse živali, ki so bile pozitivne na govejo tuberkulozo, so bile zaklane v sanitarni klavnici, njihovo mleko pa je bilo označeno kot neprimerno za prehrano ljudi. Republika Makedonija spada med države z nizko stopnjo prevalence tuberkuloze pri govedu s skupno povprečno prevalenco 0,002 % v obdobju 2007-2009. Severozahodna, jugozahodna in vzhodna področja države je bolezen močneje prizadela. Za učinkovitejši nadzor je potrebno povečati število testiranih goved, čemur morajo slediti sanitarni ukrepi in epidemiološko sledenje.

**Ključne besede:** tuberkuloza; govedo; tuberkulinski test; zoonoza; varnost hrane

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**Original Scientific Article**

# DETECTION OF SIX HONEYBEE VIRUSES IN CLINICALLY AFFECTED COLONIES OF CARNIOLAN GRAY BEE *(APIS MELLIFERA CARNICA)*

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**Summary:** This research describes the detection of six honeybee viruses in samples of clinically affected Carniolan gray bee collected between 2007 and 2009 on the territory of Slovenia. Using one-step reverse transcription-PCR (RT-PCR), 60 bee samples originated from 45 apiaries were screened for the presence of six honeybee viruses. Samples were found positive for acute bee paralysis virus (ABPV = 40%), black queen cell virus (BQCV = 83,3%), chronic bee paralysis virus (CBPV = 18,3%), deformed wing virus (DWV = 70%), Kashmir bee virus (KBV = 1,7%) and sacbrood bee virus (SBV = 8,3 %). Mortality and paralysis were often evident in the apiaries and could be connected with ABPV and/or CBPV infections. Both viruses were detected in clinically affected apiaries with high bee mortality and with paralysis symptoms showing flightless bees, trembling and crawling at the hive entrance. The severity of clinical manifestation with high bee losses were associated with higher number of viruses detected in the samples. Among virus positive samples, 27% of them were infected with one virus, 30% with two viruses, 25% with three viruses and 15% of samples contained four viruses simultaneously. The results of this study provide data about the detection of several bee viruses in affected bee colonies and viral infections in Carniolan bee have to be investigated by further research.

**Key words:** honeybee; *Apis mellifera carnica*; bee viruses, RT-PCR; diagnosis; epidemiology

## Introduction

Honeybees (*Apis mellifera* L.) are a critical player in the production of many fruit, vegetable and seed crops grown throughout the country and worth of millions euro in value to agriculture each year. The Carniolan bee *(A. mellifera carnica)* is the subspecies of the Western honey bee that has naturalized and adapted to the Slovenia geographic area, the Southern part of the Austrian Alps and North Balkan countries. It is indigenous bread and one of the most popular bee races. In Slovenia, it is protected under the law and introducing other bee species is not allowed. Carniolan bee is gentle, not aggressive, has good sense for orientation and is though less drifting to a neighbouring hives. In the winter it is able to survive with not much honey stores, which is good feature for the areas with long winters. The increased number of honeybee colonies losses in our country during the last decade has resulted great interest in honeybee pathology and viruses have emerged as one of several candidates for these losses. Viruses are probably the least understood part of honeybee pathology mainly because of the lack of information of the objective data about viral disease outbreaks. With rapid dissemination of the ectoparasitic mite varroa (*Varroa destructor)* and bee losses, viral honeybee diseases have been

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detected in Europe and worldwide in last years (1-6). When varroa is spreading among bees in the colony and between apiaries to the long distances *varroa* is considered as an important vector for many viruses. In addition because of sucking bee haemolymph, varroa suppresses its immunity (7).

The most commonly observed bee viruses are single stranded RNA viruses and include *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV) and *Kashmir bee virus* (KBV) which are classified as members of the genus *Cripavirus* (family *Dicistroviridae*), *Deformed wing virus* (DWV) and *Sacbrood bee virus* (SBV) are assigned to the genus *Iflavirus* and *Chronic bee paralysis virus* (CBPV) which also possesses an RNA genome, but is not picorna-like and remains unclassified. Five of these viruses, ABPV, DWV, KBV, SBV and CBPV can cover and over infection with clinical signs that can be identified by beekeepers, while the majority of the bee virus infections are believed to cause persistent, usually inapparent infections (8).

The great diversity of viruses isolated from honey bees, the lack of specific clinical signs and partial or complete sequencing of several RNA viruses of the honeybee has resulted in the development of several RT-PCR methods and applied for the diagnosis of ABPV, BQCV, CBPV, DWV, KBV and SBV (9-17). Clinical signs and laboratory diagnostics supported the presence of ABPV, DWV and SBV in Slovenia already in 2004 (18).

Between 2007 and 2009, the increased losses of bee colonies have been reported in Slovenia. In this paper we present the results of first survey of bee viruses, with the prevalence of six RNA viruses in samples collected in the apiaries in five different geographic regions, covering whole country. The aim of this survey was also to establish the routine laboratory molecular methods for specific detection of honey bee viruses and to obtain comprehensive insight into the correlation between the presence of honeybee viruses and their clinical manifestation in bee colonies.

## Materials and methods

Sixty samples of dead worker Carniolan bee (Apis mellifera carnica) were collected from 45 different apiaries located in five geographical regions in Slovenia (Primorska, Gorenjska, Dolenjska, Štajerska, Prekmurje). Bee samples were collected from January 2007 to December 2009 and were mostly associated with abnormal behaviour of bees, mortality or sudden colony losses. Each sample consisted of a pool of 50 to 100 bees of the same beekeeper; in few apiaries more than one sample was collected from the same apiary where clinical manifestation of the infection was present. Samples were collected by the veterinarians – specialists for bee diseases and samples were as soon as possible sent to the Virology department (National Veterinary Institute, Slovenia) where they were stored at low temperature (less than minus 60 °C) until used. For the virus analysis, 10 to 15 bees were randomly selected from each bee sample and placed into the sterile plastic bags. The samples were homogenized in RPMI (Gibco) with homogenizer (IUL masticator). After homogenization, the sample suspensions were centrifuged for 15 min at 2.500 rpm. The supernatant was recovered and 140 µl of the supernatant was used for the extraction of total RNA using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Each RNA sample was tested for the presence of nucleic acids of six viruses (Table 2). A part of viral genome of ABPV, BQCV, CBPC, DWV, KBV and SBV was amplified by RT-PCR using specific primer pair (Table 1) and One-Step RT-PCR kit (Qiagen, Germany) reagents according to the manufacturer's instructions. Reaction mixtures without RNA served as negative controls, and verified positive samples of each virus as a positive control. The reaction was performed in a total volume of 25 µl as follow: 15 µl of nuclease free water, 5 µl of 5 x PCR buffer, 1 µl of dNTP mix (containing 10 mM of each dNTP), 0,5 µl of stock solution of 20 µM of each primer, 1 µl of one step RT-PCR enzyme mix and 2 µl of RNA template. The primers used in the assay are shown in Table 1. The amplification program included reverse transcription stage at 50 °C for 30 min, followed by an initial PCR activation step at 95 °C for 15 min. This was followed by 40 cycles of 94 °C for 30 sec, 54 °C for 30 sec (for DWV and CBPV, annealing temperature was 60 and 55 °C, respectively) and 72 °C for 1 min and final extension step at 72 °C for 10 min. The reaction was performed using T1 Biometra Thermocycler and PCR products were visualized in 1,8% agarose gel with 0,5 µg/ml ethidium bromide and subsequent visualization under UV light. The size of each PCR product was compared to the 100-bp DNA ladder (Fermentas, Germany) and results for each virus were interpreted as positive or negative according

**Table 1:** Sequence of primers used in this study

| Primers (5'-3') | Position in genome | Product (bp) | Reference |
|---|---|---|---|
| Acute bee paralysis virus | | | |
| ABPV-1 (cat att ggc gag cca cta tg) | 8114 - 8512 | 398 | 11 |
| ABPV-2 (cca ctt cca cac aac tat cg) | (capsid protein) | | |
| Black queen cell virus | | | |
| BQCV-F (tgg tca gct ccc act acc tta aac) | 7850 - 8550 | 700 | 12 |
| BQCV-R (gca aca aga aga aac gta aac cac) | (structure polyprotein) | | |
| Chronic bee paraliysis virus | | | |
| CBPV1-1 (tca gac acc gaa tct gat tat tg) | 147-716 | 570 | 16 |
| CBPV1-2 (act act aga aac tcg tcg ctt cg) | (RNA polymerase) | | |
| Deformed wing virus | | | |
| DWV F (agg cga cat ggg aac agg) | 1312-1815 | 504 | 18 |
| DWV R (caa ctt cac cct cgc cat ca) | (capsid protein) | | |
| Kashmir bee virus | | | |
| KBV 1 F (gat gaa cgt cga cct att ga) | 5406 - 5820 | 414 | 8 |
| KBV 1 R (tgt ggg ttg gct atg agt ca) | (RNA polymerase) | | |
| Sacbrood bee virus | | | |
| SBV-F (gct gag gta gga tct ttg cgt) | (4957-5781) | 824 | 14 |
| SBV-R (tca tca tct tca cca tcc ga) | (structure polyprotein) | | |

to the expected size of DNA fragment (Table 1). A limited number of PCR amplicons, specific for each of six bee viruses, were purified using Wizard PCR Prep DNA Purification System (Promega) and sequenced to confirm the specificity of RT-PCR assays. The obtained nucleotide sequences of each virus were analyzed with DNASTAR program (Lasergene, USA) and compared with the published sequences in GenBank database using National Centre for Biotechnology Information (NCBI) to specify the amplicons for each PCR.

## Results

60 samples, originated mainly from the affected Carniolan bee colonies, from 45 apiaries in five Slovenian geographic regions (Primorska, Gorenjska, Dolenjska, Štajerska, Prekmurje), were collected and tested for the presence of six bee viruses by specific RT-PCR methods. In the majority of apiaries, where samples were collected, clinical signs of disease were reported with one or more symptoms confirmed by veterinarian specialists: sudden bee losses, high mortality of colonies (more than 50% of bee colonies losses in the apiary), mortality (less than 50% of bee colonies losses), bee paralysis, wing deformities, varroa infestation and affected bee brood (Table 2). All these bee colonies have had a history data with varroa infestation and were intensively treated by acaricides in 2008 and 2009. Three samples of apparently healthy bees (samples with numbers 20, 21 and 22, Table 2) were collected in the year 2009. Twenty-four samples (40%), 50 (83,3%), 11 (18,3%), 42 (70%), 1 (1,6%) and 5 (8,3%) of 60 samples were positive for ABPV, BQCV, CBPV, DWV, KBV and SBV, respectively (Table 2). The majority of bee samples (70%), collected from apiaries where bee losses, paralysis or varrosis were reported were positive for two to four different viruses (30% of bee samples contained two viruses, 25% three viruses and 15% four viruses), while in 16 bee samples only BQCV, CBPV or DWV was detected (Table 3). The severity of clinical manifes-

tations with high bee losses was associated with higher number of viruses detected in the samples (Table 2). SBV was detected in five samples from five apiaries where typical signs of the diseased sac brood were observed. When more than one sample was collected from different colonies of the same apiary, very similar number and patterns of detected viruses was observed. Two of three bee samples collected from apiaries with no symptoms of disease were positive only for BQCV and no nucleic acids of other five viruses were detected. Regional differences in the distribution of six viruses were not ascertained (Table 2). Nucleotide sequences of the amplified product were determined and confirmed the expected bee virus using BLAST at the NCBI. BLAST sequence alignment of each obtained sequence with published sequences has resulted in 96% sequence identity for ABPV (381 nucleotide-nt), 99% for BQCV (561 nt), 99% for CBPV (522 nt), 97% for DWV (471 nt), 99% for KBV (402 nt) and 95% identity for SBV (711 nt) (data not shown). According to the obtained sequence data for six bee viruses from this study the primers used for PCR amplification are specific for detection of determined bee viruses.

**Table 2:** Honeybee viruses (ABPV, BQCV, CBPV, DWV, KBV and SBV) detected in sixty bee samples from five geographic regions in Slovenia

| Sample number | Geografic area | Year of sampling | ABPV | BQCV | CBPV | DWV | KBV | SBV | Clinical symptoms | No. of viruses detected |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Primorska | 2008 | - | + | - | - | - | - | Paralysis | 1 |
| 2 | Gorenjska | 2009 | - | + | - | + | - | - | Mortality and varrosis | 2 |
| 3 | Prekmurje | 2009 | + | + | - | + | - | - | Paralysis | 3 |
| 4 | Gorenjska | 2008 | - | + | - | + | - | - | High mortality | 2 |
| 5 | Štajerska | 2008 | + | + | - | + | - | - | High mortality and varrosis | 3 |
| 6 | Štajerska | 2008 | + | + | + | + | - | - | High mortality | 4 |
| 7 | Štajerska | 2008 | + | + | + | + | - | - | High mortality | 4 |
| 8 | Gorenjska | 2008 | - | + | - | + | - | - | Mortality | 2 |
| 9 | Štajerska | 2008 | + | + | - | + | - | - | Paralysis | 3 |
| 10 | Štajerska | 2007 | + | + | - | + | - | - | High mortality | 3 |
| 11 | Štajerska | 2007 | + | + | - | + | - | - | High mortality | 3 |
| 12 | Gorenjska | 2008 | - | + | - | - | - | - | Mortality | 1 |
| 13 | Štajerska | 2007 | + | + | - | + | - | - | Mortality | 3 |
| 14 | Štajerska | 2007 | + | + | - | + | - | - | High mortality | 3 |
| 15 | Štajerska | 2007 | + | + | + | + | - | - | High mortality | 4 |
| 16 | Štajerska | 2007 | + | + | - | - | - | - | High mortality | 2 |
| 17 | Štajerska | 2007 | - | + | + | + | - | + | Mortality | 4 |
| 18 | Gorenjska | 2009 | + | + | - | + | - | - | Mortality | 3 |
| 19 | Prekmurje | 2009 | - | + | - | + | - | - | Mortality | 2 |
| 20 | Dolenjska | 2009 | - | + | - | - | - | - | No symptoms | 1 |
| 21 | Prekmurje | 2009 | - | + | - | - | - | - | No symptoms | 1 |
| 22 | Prekmurje | 2009 | - | - | - | - | - | - | No symptoms | 0 |
| 23 | Prekmurje | 2009 | + | + | - | + | - | - | Paralysis | 3 |
| 24 | Dolenjska | 2009 | - | - | + | - | - | - | Paralysis | 1 |
| 25 | Dolenjska | 2009 | - | + | - | - | - | + | Affected brood | 2 |
| 26 | Štajerska | 2009 | + | + | - | - | - | + | Mortality | 3 |
| 27 | Primorska | 2009 | + | + | - | + | - | + | Affected brood | 4 |
| 28 | Prekmurje | 2009 | - | + | - | - | - | - | Paralysis | 1 |
| 29 | Štajerska | 2009 | - | + | - | + | - | - | Mortality and paralysis | 2 |
| 30 | Prekmurje | 2009 | - | + | - | - | - | - | Paralysis | 1 |
| 31 | Prekmurje | 2009 | - | + | - | - | - | - | Varrosis and paralysis | 1 |
| 32 | Prekmurje | 2009 | - | + | - | - | - | - | Paralysis | 1 |
| 33 | Prekmurje | 2009 | - | + | - | - | - | - | Varrosis and paralysis | 1 |
| 34 | Prekmurje | 2009 | + | + | - | - | + | - | Mortality | 3 |
| 35 | Prekmurje | 2009 | - | + | - | + | - | - | Mortality and paralysis | 2 |
| 36 | Prekmurje | 2009 | - | - | - | + | - | - | Paralysis, deformed wings | 1 |

| Sample number | Geografic area | Year of sampling | ABPV | BQCV | CBPV | DWV | KBV | SBV | Clinical symptoms | No. of viruses detected |
|---|---|---|---|---|---|---|---|---|---|---|
| 37 | Štajerska | 2009 | - | + | - | + | - | - | Varrosis | 2 |
| 38 | Štajerska | 2009 | - | + | - | + | - | - | Varrosis | 2 |
| 39 | Štajerska | 2009 | + | + | - | + | - | - | Mortality and paralysis | 3 |
| 40 | Gorenjska | 2009 | - | - | - | + | - | - | Mortality | 1 |
| 41 | Gorenjska | 2009 | + | + | - | + | - | + | High mortality | 4 |
| 42 | Gorenjska | 2009 | + | - | - | + | - | - | Mortality and varrosis | 2 |
| 43 | Gorenjska | 2009 | + | - | - | + | - | - | High mortality | 2 |
| 44 | Štajerska | 2009 | - | + | - | - | - | - | Mortality | 1 |
| 45 | Štajerska | 2009 | - | - | - | - | - | - | Mortality | 0 |
| 46 | Štajerska | 2009 | + | + | + | + | - | - | Mortality and varrosis | 4 |
| 47 | Štajerska | 2009 | - | + | - | + | - | - | Mortality and varrosis | 2 |
| 48 | Gorenjska | 2009 | - | + | - | + | - | - | Mortality | 2 |
| 49 | Gorenjska | 2009 | - | + | - | + | - | - | Mortality | 2 |
| 50 | Gorenjska | 2009 | - | + | - | + | - | - | Mortality | 2 |
| 51 | Štajerska | 2009 | + | + | + | - | - | - | Mortality and paralysis | 3 |
| 52 | Gorenjska | 2009 | - | + | + | + | - | - | Mortality and varrosis | 3 |
| 53 | Gorenjska | 2009 | + | + | + | + | - | - | Mortality and varrosis | 4 |
| 54 | Gorenjska | 2009 | + | + | + | + | - | - | Mortality and varrosis | 4 |
| 55 | Gorenjska | 2009 | - | - | - | + | - | - | Mortality and varrosis | 1 |
| 56 | Gorenjska | 2009 | - | + | + | + | - | - | Mortality and varrosis | 3 |
| 57 | Gorenjska | 2009 | - | + | - | + | - | - | Mortality and varrosis | 2 |
| 58 | Primorska | 2009 | - | - | - | + | - | - | High mortality | 1 |
| 59 | Primorska | 2009 | - | - | - | + | - | - | Mortality | 1 |
| 60 | Prekmurje | 2009 | - | + | - | + | - | - | High mortality | 2 |
| Number (%) of positive samples | | | 24 (40) | 50 (83,3) | 11 (18,3) | 42 (70) | 1 (1,7) | 5 (8,3) | | |

Legend: - RT-PCR negative result, + RT-PCR positive result, high mortality (more than 50 % of bee colonies losses reported in the apiary), mortality (less than 50 % of bee colonies losses reported in the apiary).

**Table 3:** The occurrence of virus infections in Carniolan honeybee samples

| No. of viruses | Detected viruses | No. of samples | Percent of samples |
|---|---|---|---|
| 0 virus | - | 2 | 3,3% |
| 1 virus | BQCV or CBPV or DWV | 16 | 26,7% |
| 2 viruses | BQCV, CBPV, DWV | 18 | 30% |
| 3 viruses | ABPV, BQCV, CBPV, DWV | 15 | 25% |
| 4 viruses | ABPV, BQCV, CBPV, DWV, SBV | 9 | 15% |
| 5 viruses | - | 0 | 0% |
| 6 viruses | - | 0 | 0% |
| | Total | 60 | 100% |

## Discussion

The honeybee colonies losses worldwide during the last decade increased the interest of bee toxicology and bee pathology. In the last few years, the diagnostic methods for the honeybee viruses changed from serological to PCR-based methods.

In this research article we describe the molecular-genetic evidence of six viruses in the samples of the Carniolan gray bee collected in Slovenia in the years 2007-2009. Of the six viruses identified by RT-PCR, BQCV had the highest prevalence (83,3%), which supports published data that BQCV can persist in the colony as an unap-

parent infection in adult bees for a longer period. BQCV affects mainly developing queen larvae and capped brood. Bailey and co-workers (19) reported that infections of bees with BQCV were strongly associated with the infestation of the protozoan *Nosema apis*, a parasite of honey bees. Although adult bees are often infected with BQCV, they normally do not exhibit the disease symptoms.

With the 70% prevalence, DWV was the second most prevalent virus. The veterinarians did not often report about typical symptoms for the DWV infection, such as shrunken, crumpled wings, reduced size and discoloration of bees but they described the symptoms of paralysis and high mortality which could be associated with the detection of other two viruses, ABPV and CBPV. High prevalence of DWV infection in the Carniolan bee is similar to the prevalence reported previously in other bees (3, 20).

Mortality and paralysis were often evident in the apiaries with ABPV and/or CBPV infections. Both viruses were detected in clinically affected apiaries where flightless bees, trembling and crawling of bees at the hive entrance were observed. Also the observations of black and hairless bees rejected by the healthy ones were reported by our veterinarians. In several affected Carniolan colonies, the bees left the queen with few workers or the bees were unable to fly and died within few days in thousands. ABPV was detected in the apiaries in some European countries and with its high prevalence of 40% is widespread also in Slovenia. In France 58% (20) of apiaries were infected with ABPV, in Hungary 67% (11) and in Austria 68% (3).

In our study SBV, was detected rarely (8,3%) only in adult bees or larvae. Much higher (86%) SBV prevalence was reported in France (20), 48% in Austria (3) and 100% in Uruguay (21). In Slovenia all five SBV positive cases were detected in the apiaries where the clinical manifestation of sac brood disease was recorded. This is in agreement with previous observations that this virus infection can be readily and easily diagnosed by the typical clinic symptoms of the honeybee brood disease.

KBV was detected only in one sample out of sixty tested samples in our study. It has already been reported that this virus is less prevalent in the cases where many viruses were present in the same honeybee population (1-3, 20, 22).

In two previous studies in Slovenia 24 and 4 samples had already been examined for the presence of DWV, ABPV, SBV and KBV in 2004 (18) and 2006 (3) respectively. In both studies ABPV, DWV and SBV were identified from randomly collected samples. In this study almost all collected samples were obtained from the colonies with clinical symptoms of bee disease and from the previously published data, the detection of bee viruses was expected. However, three viruses previously not detected in Slovenia were confirmed in our study. Surprisingly, 70% of examined bee samples had more than one virus present at the same time suggesting that many honeybee viruses are widely represented on our territory. Among all virus positive samples, 27% of them were infected with one virus, 30% with two viruses, 25% with three viruses and 15% of samples contained four viruses simultaneously. The results of this study provide the evidence of the detection of several bee viruses in affected Carniolan bee colonies and these viruses have to be investigated by further research. However, it is evident that the majority of Slovenian apiaries with affected bee colonies have multiple virus infections. Another study already confirmed that different viruses could be detected also in a single bee, in the colony (14). Ninety-two percent of the apiaries in France were found positive for at least three different viruses (31% of the apiaries contained three viruses, 36% four viruses and 25% five viruses) (20). The percentage of multiple virus infections in France apiaries was even higher than it was determined in our study.

Although the detection of varroa mite was not a specific topic of our study, its significance in horizontal transmission of viruses among bees could not be ignored. Honeybees have non-specific defence system as mechanical barriers with cuticle and hair and the non specific immune system. With varroa introduction, this system fails because the varroa sucks haemolymph and cause anaemia with reducing immune system, perforate the cuticle and introduce viruses. High varroa infestations could affect the virus distribution in bee colony (5, 20). Varroa suck the bee haemolimph with their strong mouth apparatus and directly injects viruses (ABPV, BQCV, DWV, KBV, SBV) in the bee body. The finding of bee viruses in varroa strongly supports the important role of varroa in the viral infections of bees (20, 23). In Slovenia, the national varroa treatment campaign started in 2009 lowered the varroa infestation and probably prevented even harder colony losses. However, the treatment with acaricides had limited success be-

cause of possible resistance and reintroduction of varroa. In our research we found that many apiaries still suffered from varrosis in the year 2009 (Table 2). In conclusion, we confirmed that the varroa is present in our area as elsewhere, it plays an important role in the honeybee pathology and it is together with different bee viruses one of the main causative agent for the bee losses.

The apiaries, which are infested with varroa and co-infected with ABPV, BQCV, CBPV and DWV as a single or multiple infections could potentially be at high risk for the colony losses. The experiments done on the artificially inseminated queens with the semen originated from the brothers of the drones, from which virus-positive semen was collected, showed that the transmission of viruses through sperm in most cases do not overt signs of the disease (24). Another data demonstrated that queens can harbour multiple viruses, although they show no clinical signs of the infection (1). This supports the theory of co-evolution of the honey bees and the viruses which has resulted in a balance allowing both partners to survive. However the clinical manifestation of the virus infections in the honey bee colony has dramatically changed when varroa emerged and started to spread the viruses among the bee colonies with varroa infested drifting bees. This new route of virus transmission probably resulted in the majority of detected bee losses between 2007 and 2009. The need for the development of new methods such as multiplex RT-PCR and real – time PCR reported by other authors also applies to our laboratory. These methods will allow the simultaneous detection of different viruses in a single reaction, allowing us to screen honeybee viral infections in a large number of samples within short time and less money.

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## DOKAZ ŠESTIH ČEBELJIH VIRUSOV V ČEBELJIH DRUŽINAH KRANJSKE ČEBELE *(APIS MELLIFERA CARNICA)* S KLINIČNO SLIKO OBOLENJA

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**Povzetek:** Raziskava opisuje dokaz šestih čebeljih virusov v vzorcih klinično obolelih čebeljih družin kranjske čebele zbranih na področju Slovenije med letoma 2007 in 2009. Z uporabo metode reverzne transkripcije in verižne reakcije s polimerazo (RT-PCR) smo na šest čebeljih virusov pregledali 60 vzorcev čebel, ki so izvirali iz 45 čebelnjakov. Virus akutne paralize čebel (ABPV) smo dokazali v 40 % vzorcev, virus črnih matičnikov (BQCV) v 83,3 % vzorcev, virus kronične paralize čebel (CBPV) v 18,3 % vzorcev, virus deformiranih kril (DWV) v 70 % vzorcev, kašmirski virus čebel (KBV) v 1,7 % vzorcev, virus mešičkaste zalege (SBV) pa v 8,3 % od pregledanih vzorcev. V čebelnjakih smo ob odvzemu vzorcev pogosto zabeležili smrtnost in paralizo čebel, ki bi ju lahko povezali z laboratorijsko ugotovitvijo okužb z ABPV in CBPV. Oba virusa smo dokazovali pri klinično prizadetih čebeljih družinah skupaj z zabeleženo visoko smrtnostjo in z znaki paralize pri čebelah, zaradi česar čebele niso bile sposobne leteti in so umirale na tleh pred čebelnjakom. Intenzivnost opisane klinične slike z opisanimi izgubami čebel v čebelnjaku se stopnjujejo z ugotovitvijo večjega števila dokazanih različnih virusov v vzorcu. Med pozitivnimi vzorci smo pri 27 % ugotavljali okužbo z enim virusom, pri 30 % vzorcev okužbo z dvema različnima virusoma, pri 25 % vzorcih s tremi virusi, 15 % vzorcev pa je sočasno vsebovalo štiri različne viruse. Z rezultati te študije smo dokazali številne viruse v vzorcih prizadetih čebeljih družin, zato je potrebno virusnim okužbam kranjske čebele v prihodnje nameniti posebno pozornost.

**Ključne besede:** medonosna čebela; *Apis mellifera carnica*; virusi pri čebelah, RT-PCR; diagnostika; epidemiologija

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**Original Scientific Article**

# THE EFFECT OF DIFFERENT DILUTION RATES ON POST-THAW QUALITY OF RAM SEMEN FROZEN IN TWO DIFFERENT EGG-YOLK FREE EXTENDERS

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**Summary:** The objective of the present study was to evaluate the effects of pre-freezing sperm concentration (200, 400 or 800x$10^6$ spermatozoa/ml) using two commercial extenders (Bioexcell® and Andromed®) on post-thaw survival and acrosomal status of ram spermatozoa. Semen samples were obtained from the 5 mature Karayaka rams (aged 2-3 yr) and a total of 30 ejaculates collected from each male twice a week for 3 weeks with the aid of an artificial vagina, during the non-breeding season (February, winter). Semen extended in Bioexcell® or Andromed® diluents, was loaded into 0.25 ml straws and equilibrated at 4°C for 2 h. Straws were frozen in the vapour of liquid nitrogen and then stored at -196°C. After thawing (at 37 °C for 30 sec), sperm motility, acrosomal status and membrane integrity were assessed. Pre-freezing sperm concentration influenced ($P<0.001$) frezability of spermatozoa and affected all the in vitro parameters at 400x$10^6$ and 800x$10^6$ spermatozoa/ml negatively regardless of the extender. Decreasing the sperm concentration into 200x$10^6$ spermatozoa/ml influenced positively the percentage of sperm motility and membrane integrity extended in Bioexcell® (40 %, 29 %) and Andromed® (43 %, 32 %). The lowest percentage of abnormal acrosome was also described at lowest sperm concentration (200x$10^6$ spermatozoa/ml) in both extenders as 29 and 26 %. It was concluded that significant differences exist between the dilution rates or sperm concentrations. Lower sperm concentrations or higher dilution rates with the commercial extenders were better to protect sperm from damages during cryopreservation.

**Key words:** ram; semen; dilution rate; commercial extender; cryopreservation

## Introduction

Egg yolk is a main component in the extenders for storage and cryopreservation of semen in most mammalian species including bull, ram, goat. The main effective component of egg yolk is the low density lipoprotein fraction like lecithin, which protects the membrane phospholipid integrity during cryopreservation (1). However, in recent years, there has been frequent opinion against the use of egg yolk due to the wide variability of its constituents, which makes evaluation of its beneficial component complex. Furthermore, egg yolk increases the risk of microbial contamination and thereby allows subsequent production of endotoxin, which may reduce after thawing viability and acrosome integrity of spermatozoa in some species such as ram, goat, and buffalo (2-8). Therefore, it would be preferable to use the diluents free from egg yolk. Andromed® and Bioexcell® is a commercially available extender without components of animal origin. It contains vegetal lecithin as a cold shock protector, and has been used for freezing buck and ram semen with satisfactory results (9, 10, 11).

The survival of frozen–thawed ram sperm is affected by many factors, extensively reviewed by Salamon and Maxwell (12). The rate of semen dilution is one factor determining cryopreservation

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success but there are few comparative studies on the effects of sperm concentration for freezing ram semen (13). The rate of dilution is usually varied to produce a standardized number of spermatozoa per inseminate dose or is simply based on the number of females to be inseminated per ejaculate (12). Early researchers employed dilution rates ranging from 2 to 5 fold, which are significantly higher than the 10–15 fold dilution rates commonly used today (12). Excessive dilution has been reported to cause membrane destabilization and capacitation-like changes in spermatozoa and cryopreservation may have an additive effect, further injuring the cells. It is thought the "dilution effect" is due to the removal of protective factors in seminal plasma (14). However, there is a lack of information on the pre-freezing rate to which spermatozoa can be diluted without a reduction in their post-thaw survival. Moreover, there are no studies concerning the effect of Bioxcell® and Andromed® extenders on ram semen freezability.

The aim of the present study was to evaluate the effects of three different pre-freezing dilution rates on sperm motility, acrosome abnormality, and plasma membrane integrity, in Karayaka ram semen, frozen in egg yolk free commercial extenders, Andromed® and Bioexcell®.

## Materials and methods

### *Animals and semen collection*

Five mature Karayaka rams (aged 2-3 years) with proven fertility were housed at the Education Research and Practice Farm, Faculty of Veterinary Medicine, University of Ankara, Turkey at 39 ° 57′ N, 32 ° 53′ E, at an altitude of 850 m. The rams (65-70 kg) were kept under natural light and maintained under a uniform and constant nutrition regime with each ram being fed a daily diet of 1 kg concentrate, dried grass, salt lick and water ad libitum. A total number of 30 ejaculates were collected using artificial vagina from each male twice a week for 3 weeks during the nonbreeding season (February, winter).

### *Semen extenders*

Two extenders, Bioxcell® (Extender B) and Andromed® (Extender A), were used for the dilution of semen in the present study. The extenders were prepared as follows:

Extender B: A commercially available diluent (IMV, Aigle, France). This extender contains soybean extract with antibiotics (lincomycin, spectinomycin, tylosin, gentamycin) and glycerol (7%).

Extender A: A commercially available diluent (Minitüb, Tiefenbach, Germany). This extender contains: bi-distilled water, fructose, glycerol, citric acide, buffers and phospholipids with antibiotics (lincomycin, spectinomycin, tylosin, gentamycin) and glycerol (6.7%).

### *Semen dilution, freezing and thawing*

Only ejaculates with a minimum concentration of $3x10^9$ spermatozoa/mL and 70% progressively motile cells were pooled across rams. The volume of semen ejaculates were measured in a conical tube graduated at 0.1 ml intervals and the sperm concentration was estimated using a haemocytometer (15). Semen extended Bioexcell® or Andromed® in diluent to a final concentration of 200, 400 or $800x10^6$ spermatozoa/ml was loaded into 0.25 ml plastic straws (IMV, Laigle, F-61300, France) and sealed with polyvinyl alcohol (PVA). Straws were equilibrated at 4 °C for 2 hr and after equilibration, the straws were suspended on a styrofoam rack 4 cm above the liquid nitrogen (vapour) for 15 min. The straws were plunged into the liquid nitrogen; where stored until thawing. After storage for a period of 3 weeks, the semen straws were thawed in a water bath (37 °C for 30 s) for microscopic semen evaluation immediately after thawing. The experiment was conducted in six replicates.

### *Semen evaluation*

Sperm motility was evaluated subjectively using a phase-contrast microscope (400x), with a warm stage maintained at 37°C. A wet semen mount was made using 5 µL semen placed directly on a microscope slide and covered by a cover slip. For each sample, at least 5 microscopic fields were examined by 3 trained observers. The mean of the three successive evaluations was recorded as the final motility score (5).

For the assessment of acrosomal abnormalities, at least three drops of each sample were added to an Eppendorf container containing 1 mL Hancock solution (62,5 mL formalin, 37%), 150 mL saline solution, 150 mL buffer solution and 500 mL double-distilled water) (16). One drop of this

semen mixture was put on a slide and covered with a cover slip. The percentage of the acrosomal abnormalities was determined by counting a total of 200 sperm under phase-contrast microscope using an immersion objective.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, by incubating 100 µL semen with 1000 µL of a 100 mOsm hypo-osmotic solution (9 g fructose and 4.9 g sodium citrate per liter of distilled water) at 37°C for 30 min. After incubation, 5 µL of the mixture was spreaded with a cover slip on a warm slide. A total of 200 spermatozoa were counted in different fields at 400x under phase contrast microscope and percentage of spermatozoa positive to HOS test (having coiled tails) was determined (17).

*Statistical analysis*

The study was repeated 6 times and the results were expressed as mean±SEM. One-way analysis of variance (ANOVA) with a subsequent Tukey's test was used to compare the mean values resulting from the various treatments at a significance level of $p < 0.05$. All analyses were carried out using the SPSS 11 for Windows statistical software package.

## Results

The effects of three different pre-freezing dilution rates on sperm motility, acrosome abnormality, and plasma membrane integrity, in Karayaka ram semen frozen in egg yolk free commercial extenders, Andromed® and Bioexcell® are presented in Table 1. Pre-freezing sperm concentration influenced ($p<0.05$) freezability of spermatozoa and affected negatively all the *in vitro* parameters at $400x10^6$ and $800x10^6$ spermatozoa/ml dilution rates regardless the extender type. The decrease of sperm concentration to $200x10^6$ spermatozoa/ml influenced positively the percentage of motility and the membrane integrity; extended in both commercial extenders. The proportion of spermatozoa with intact acrosome was influenced by either pre-freezing sperm concentration or extender. The highest proportion of spermatozoa with intact acrosomes were observed in samples frozen with Bioxcell® and Andromed® at the $200x10^6$ spermatozoa/ml compared to $400x10^6$ and $800x10^6$ spermatozoa/ml.

## Discussion

These results demonstrate that there is a considerable reduction in ram sperm motility, acrosome and membrane functionality following cryopreservation at low sperm dilution rates or with increased numbers of spermatozoa per dose. Conforming the results of similar studies (13, 18).

A commercially available extender in which egg yolk is substituted by soybean lecithin has been recently tested in different species (19, 20, 21). Fukui et al. (9) reported that Andromed® rendered fertility results comparable to egg yolk extenders, after intrauterine insemination of sheeps. This makes Andromed® a promising option for further research on sheeps. And also Bioexcell® is recommended due to its animal ingredient free properties. It was developed for bovine semen cryopreservation and has been applied to other animal species including

**Table 1:** Effect of different dilution rates on motility, acrosomal abnormality and plasma membrane integrity after thawing (n=6)

| Extender | Sperm Concentration ($x10^6$sperm/ml) | Motility (%) | Acrosomal abnormality(%) | Plasma membran integrity (%) |
|---|---|---|---|---|
| Bioxcell® | 200 | 40±3.5a | 29±2.1a | 29± 3.1a |
| | 400 | 20±1.5b | 40±2.5b | 18±1.9b |
| | 800 | 13±1.2b | 48±2.6b | 14±2.0b |
| Andromed® | 200 | 43±4.5a | 26±3.5a | 32±2.8a |
| | 400 | 30±2.5b | 34±2.0b | 22±1.7b |
| | 800 | 21±2.8b | 44±2.3b | 19±1.4b |

a,b: Different superscripts in the same column indicate significant differences ($p<0.05$).

sheep and goat (10, 11). Gil et al. (10) reported that ram spermatozoa in soya lecithin based extender Bioxcell® maintained the sperm quality and produced acceptable fertility rates.

One of the fundamental steps of semen manipulation is described by the dilution rate according to a constant concentration of spermatozoa in the current study. Post-thawing characteristics of spermatozoa were significantly impaired by increase of prefreezing sperm concentration to 400 and 800x10$^6$ spermatozoa/mL.

D'Alessandro et al. (13) also found a positive effect of high pre-freeze extension of ram spermatozoa, where the highest post-thaw motility and acrosome integrity was observed when semen was diluted to 200 million spermatozoa per ml and lowest after dilution to 800 million per ml. These results agree with the idea of better post-thaw seminal characteristics in lower sperm concentrations. Samper and Morris (22) suggested that freezing at lower sperm concentrations may initially provide a higher availability of nutrients and increase cryoprotectant property per spermatozoon, which may explain the higher percentage of motile spermatozoa immediately after thawing when frozen at 100x10$^6$ ml. In parallel, Leahy et al. (18) reported that ram spermatozoa may be extended at high rates prior to freezing, at least to 20 million cells per mL, but not post-thaw. Dilution prior to freezing improved motility, viability and acrosome integrity when assessed over 6 h of incubation period.

A dose of 25–50x10$^6$ sperm for laparoscopic intrauterine insemination, 75-100x10$^6$ sperm for transcervical insemination and 150-300x10$^6$ for cervical insemination is recommended in ewes (23). In the present study, three concentrations (200x10$^6$, 400x10$^6$ and 800x10$^6$) of spermatozoa were selected to assess the changes after thawing. The changes in the sperm structure function and motility parameters were less dramatic in the 200x10$^6$ ml compared to 800x10$^6$ and 400x10$^6$ ml. The possible physiological reasons for the decline might be due to extracellular oxidative stress, effects of seminal plasma volume-constituents and endogenous free radical production. Substances from seminal plasma protect spermatozoa from premature aging during storage (24). It is suggested that the amount of seminal plasma surrounding each spermatozoon in an ejaculate varies among different concentrations (25). Along with this fact, the higher volume of extender and its contents may be one of the reasons for better preservation of functional and motility parameters at the lower concentration in our study. In this way, lower sperm concentration increased the percentage of intact plasma membrane and acrosome integrity, similar to the effect on post-thaw seminal characteristics. Possibly the frozen spermatozoa in 200x10$^6$ ml had a higher ratio of cryoprotectant agents per cell than 400x10$^6$ ml and 800x10$^6$ ml at a higher sperm concentration. The higher the amount of cryoprotectant per sperm cell, possibly the higher the percentage of unfrozen water channels, leading to better post-thaw seminal characteristics (26).

To conclude, commercial extenders seems to be useful as an alternative to the conventional extenders (Tris based, Milk Based) at higher dilution rates for the freezing of ram semen. The results of this study only reveal that these two extenders are suitable for cryopreservation of ram semen for in vitro use. In order to evaluate the use of the extenders for in vivo use, further studies are necessary.

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# VPLIV KONCENTRACIJE OVNOVEGA SEMENA NA NJEGOVO KAKOVOST PO ZAMRZOVANJU V DVEH RAZLIČNIH RAZREDČEVALCIH BREZ JAJČNEGA RUMENJAKA

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**Povzetek:** Namen dela je bil oceniti vpliv koncentracije ovnovega semena pred zamrzovanjem (200, 400 oz. 800 milijonov semenčic/ml) z uporabo dveh komercialnih razredčevalcev (Bioexcell ® in Andromed ®) na preživetje semenčic in stanje njihovega akrosoma. Vzorci semena so bili pridobljeni od 5 spolno zrelih ovnov pasme karajaka, starih 2-3 leta. Skupno je bilo s pomočjo umetne nožnice odvzetih 30 ejakulatov. Odvzem smo opravili v treh tednih v neparitvenem obdobju (februar), vsakemu ovnu pa smo seme odvzeli dvakrat tedensko. Seme smo razredčili z razredčevalcem Bioexcell ® ali Andromed ® in ga spravili v 0,25 ml slamice za zamrzovanje. Najprej smo ga ohladili na 4 °C za 2 uri, nato pa ga zamrznili v pari tekočega dušika in ga shranili pri -196 °C. Po odmrzovanju (pri 37 °C za 30 sekund) smo ocenili gibljivost semenčic, stanje akrosoma in celovitost membrane. Ugotovili smo, da koncentracija semena pred zamrzovanjem vpliva na uspešnost zaščite semenčic ($p < 0,001$). Koncentraciji 400 milijonov in 800 milijonov semenčic/ml sta neodvisno od razredčevalca negativno vplivali na preživetje semenčic. Zmanjšanje koncentracije semena na 200 milijonov semenčic/ml je pozitivno vplivalo na gibljivost semenčic in celovitost membrane v razredčevalcih Bioexcell ® (40 %, 29 %) in Andromed ® (43 %, 32 %). Pri najnižji koncentraciji semena (200 milijonov semenčic/ml) smo neodvisno od razredčevalca opazili tudi najnižji odstotek nenormalnih akrosomov (29 % in 26 %). Iz naših rezultatov lahko povzamemo, da so semenčice bolj zaščitene pred poškodbami med zamrzovanjem pri nižjih koncentracijah semena oz. višji stopnji redčenja s komercialnimi razredčevalci.

**Ključne besede:** oven; redčenje; komercialni razredčevalci; zaščita med zamrzovanjem

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**Original Scientific Article**

# CANINE LEISHMANIOSIS (*LEISHMANIA INFANTUM*) IN SLOVENIA: A QUESTIONNAIRE-BASED SURVEY

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**Summary:** Leishmaniosis is a disease caused by different species of a protozoan parasite of the genus *Leishmania* but the most common and broadly studied in dogs is the infection with the species *Leishmania infantum*, which is also transmissible to humans. The primary objective of this study was to obtain an epidemiological estimate on canine leishmaniosis in Slovenia during the period from 2005 to 2010. Questionnaires were sent to 105 slovenian veterinary practices, 49 responded and amongst them, 42 (85,7 %) didn't diagnose a case of leishmaniosis in a dog in the past five years. Slovenian veterinarians observed, that the canine leishmaniosis case numbers were descending or at least stagnating during the estimated period. All dogs, diagnosed to have leishmaniosis, were imported from endemic regions, mostly from Spain and France and occasionally from Portugal, Italy, Croatia and Africa. Constantly to frequently skin lesions, such as alopecia, exfoliative dermatitis, ulcers, nodules, pyoderma, lesions on the bridge of the nose (depigmentation, ulcers) and lesions on footpads (excessive nail growth, hyperkeratosis, ulcers) were observed. Eye and eyelid lesions like periocular alopecia, nodules at the edge of the eyelid, conjunctivitis and uveitis and beside these, non-dermatological signs such as apathy, elevated body temperature, anaemia, weight loss and diarrhea were frequently observed. One third of Slovenian veterinary practices had euthanized their cases in spite of the treatment and they have decided for euthanasia most often because of chronic renal failure (CRF) and zoonotic potential. Results of this survey showed that at least until 2010, Slovenia cannot be considered as an enzootic area. However an increase of travelling Slovenian dogs to the other Mediterranean countries may result in an increase of the probability of the diagnosis on imported cases. Utilization of diagnostic tests such as IFAT and PCR on a broad population of healthy and diseased dogs, together with vector analysis is proposed for the future studies.

**Key words:** leishmaniosis; dogs; *Leishmania infantum*; zoonosis; sand fly

## Introduction

Leishmaniosis is a disease caused by different species of a protozoan parasite of the genus *Leishmania* (1). This genus includes approximately 30 species of which 20 are pathogenic to humans (2) and at least 10 to dogs (3). In dogs, the most common and broadly studied is the infection with the species *Leishmania infantum* (2), which is also transmissible to humans (4). The transmission is due to infective bites of sandflies (dipterans phlebotominae). For each species of *Leishmania* only a limited number of species of these insects are able to ensure the development of the parasite and the transmission. Dogs are considered as the main reservoir of this infection for humans (5). Infection with this species commonly produces the most severe form of the disease in dogs as well as in people, so called visceral form, which is often fatal, especially if left untreated (6, 7). Visceral form of human leishmaniosis caused by *L. infantum* has been continuously present in Spain, Greece, Italy, Portugal and south of France with about 300 - 400 new human cases annually reported in the southern regions of Europe (4,8). Climate changes

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in the last decade could have enabled the formation of new endemic regions throughout Europe that are located north of the existing ones (9,10).

The lesions may concern virtually every tissue but most common clinical signs in dogs include skin lesions (11), swollen lymph nodes (12) and lesions in the eyes (13), and the most common cause of death is kidney failure (14). The diversity of clinical signs has been recently reviewed (15).

A diagnosis is made by parasitological, serological and molecular tests (16). Cytological examination of damaged and altered tissue and the extraction of pathogen's DNA from these tissues with polymerase chain reaction (PCR) are two of the most reliable among the methods detecting the parasite (17). Quantitative serology combined to a complete clinical and biochemical panel is the most relevant technique for an accurate prognosis and the choice of the best therapeutic approach (5).

Current treatment protocols in veterinary medicine include meglumine antimoniate or miltefosine, combined to allopurinol (7). These drugs can be used to improve or control the clinical condition of the dog, but do not eliminate infection (18).

Prevention of disease transmission is based mostly on the reduction of risk of exposure to insect bites (areas, period of the year, period of the day) but mainly on the use of repellents and insecticides with demonstrated preventive efficacy (pyrethroids) (19); however a lot of funding and research has recently been put into developing a vaccine (20). A vaccine is now available in some European countries.

The primary objective of this study was to obtain an epidemiological estimate on canine leishmaniosis in Slovenia during the period from 2005 to 2010. Slovenia is small EU country located below the Alps but sharing the coast of the Adriatic Sea with Italy and Croatia. Since both neighbouring countries belong to the endemic area (9, 21) there is the reasonable concern about Slovenia. We hypothesized that Slovenia haven't belong to endemic region. Climate at Slovenian coast has traditionally been sub Mediterranean, while majority of the state has been sharing continental and alpine climate. Although the temperatures associated to these climates do not seem favourable, an extension of potential vectors has been already observed in Italy both in the northeast and in the north through alpine valleys toward close to Switzerland (9). We hypothesized that veterinarians in Slovenia have already had diagnosed leishmaniosis in dogs, imported from endemic areas.

## Material and methods

### *Questionnaires*

Veterinary practices in Slovenia with at least partial practice in small animal medicine (n = 105) were included into the study. Each of them received one questionnaire designed to be filled by one practice. Confidentiality of collected data was provided. The study lasted from beginning of May 2010 until the end of October 2010. The data were collected on dogs only, using a questionnaire, which had already been used in other European countries doing the same epidemiologic research (France, Portugal, Greece, Spain, Italy) under the guidance of one of the authors (P.B). Questionnaire was translated to Slovene language and adapted with slight modifications and consisted of 8 sections:

1. general data on veterinary practice
2. importance of leishmaniosis for the practice
3. clinical signs
4. diagnosis
5. treatment
6. follow up
7. prognosis
8. prophylaxis
9. public health

Even the vets, who had never diagnosed a single case, were able (and were asked) to give answers to sections, numbered 1, 8 and 9. This way, we were able to collect more data.

### *The course of the events*

Questionnaires were sent to the practices by post at the beginning of May 2010. An invitation letter and prepaid envelope were added to the questionnaire. The address on the envelope was of Veterinary faculty of Ljubljana where returned questionnaires were collected. Confidentiality of collected data was provided. By the end of June 2010 a phone survey was combined to increase the number of answers. We found out that many vets didn't understand they were welcome to participate to the study even if never diagnosed a case

resulting in a loss of questionnaires at this point. By the request they were sent again via mail or E-mail. Returned questionnaires had been analyzed each time and responses of the practices had been evaluated.

In September 2010 a third call and sending of the questionnaires to the practices was made and at the end of October 2010 questioning was closed by receipt of 49 questionnaires.

## Results

Questionnaires were sent to 105 veterinary practices in Slovenia with 49 (46,7 %) responders. Amongst the latter, 42 (85,7 %) didn't diagnose a case of leishmaniosis in a dog in the past five years.

In seven Slovenian veterinary practices veterinarians diagnosed at least one CanL case in the period between 2005 and 2010 and estimated that the number of cases was staying the same (50 %) or getting lower (50 %). All responders shared the opinion of not being practicing in an enzootic area (not having diagnosed autochtonous cases) since they diagnosed leishmaniosis only in dogs which have been imported mostly from Spain and France or occasionally from Portugal, Italy, Croatia and Africa.

The clinical signs observed are indicated in table 1.

Slovenian veterinarians most frequently suspected leishmaniosis according to the results of blood tests as CBC and biochemistry profile. Less of the practices have used urinalysis. One prac-

**Table 1:** Symptoms of dogs with leishmaniosis, diagnosed in Slovenia in the period between 2005 and 2010 (number indicates the number of clinical practices)

| Frequency of observation / symptom | Never | Seldom | Periodically | Frequently | Very frequently | Constantly |
|---|---|---|---|---|---|---|
| Apathy, lethargy | | 1 | | 3 | 1 | 2 |
| Fever | | 1 | 1 | 2 | 1 | 1 |
| Anemia | | | 1 | 1 | 2 | 1 |
| Weight Loss | | | 1 | 3 | 2 | 2 |
| Alopecia | | | 3 | 2 | | |
| Exfoliative dermatitis with large scales | | 1 | 2 | 3 | 1 | |
| Exfoliative dermatitis with small scales | | | 2 | 2 | | |
| Skin ulcers | | 1 | 1 | | 1 | 1 |
| Pyoderma | | | 3 | 2 | | |
| Skin depigmentation | | 2 | 4 | | | |
| Skin nodules | | 2 | 2 | 1 | 1 | |
| Onychogryphosis | | | 3 | | 1 | |
| Nasal depigmentation, ulceration | | 1 | 3 | 1 | | |
| Footpads depigmentation, ulceration | | | 3 | | 1 | 1 |
| Ocular lesions | | 1 | 1 | | 2 | 1 |
| epistaxis | | 2 | 1 | | 1 | |
| Diarrhea | | | 3 | 1 | 2 | 1 |
| Limphadenopathy | | | 1 | 1 | 3 | |
| Renal failure | | | 2 | 2 | 1 | |
| Polyarthritis | | | 4 | | | |
| Osteomyelitis | | 1 | 3 | | | |

Comments to table 1: Every number in the table represents answer of one veterinary practice. Concurrently to leishmaniosis, anaplasmosis has been diagnosed by one veterinary practice in two Leishmania-infected (CanL) dogs.

tice has frequently used protein electrophoresis. Antinuclear antibody test has been periodically used by one practice while Coombs test has never been used amongst nonspecific tests.

Slovenian veterinarians have used enzyme-linked immunosorbent assay (ELISA) for *Leishmania* antibody titer determination most often to confirm the diagnosis. Two practices have used rapid tests (SNAP® Leishmania Test; IDEXX Laboratories) and PCR and one has used histopathology, immunofluorescent-antibody test (IFAT) or lymph node biopsy to confirm the diagnosis. Majority of the practices have never used fine needle biopsy of the skin, lymph nodes, bone marrow or spleen to provide materials for citology. Slovenian veterinary practices have sent materials for specific tests to the next specialized laboratories: Institute for microbiology and parasitology of Veterinary faculty of Ljubljana (50 %), Human national laboratory (10 %) and private laboratories like Laboklin (Austria) and Invitro (Austria).

## *Therapy*

A list of drugs was proposed in the questionnaire and their use indicated in the table 2

Majority of the practices have used allopurinol as a single agent to treat canine leishmaniosis. Glucantime has periodically been used only by one practice and miltefosine was not utilized (this molecule was not available on the veterinary market before 2007 and was launched only in some European countries not including Slovenia). Several other medicaments, like quinolones and azoles have been used by certain practices on regular basis. Occasionally pain at application site has been observed with antimoniate treatment and the appearance of crystals in the urine has been noticed with allopurinol treatment. Reported side effects haven't led to drug discontinuation.

Half of the practices that have treated CanL stopped their treatment on the basis of clinical improvement and a quarter of practices on the basis of protein analysis. For the rest, criteria stood unknown. In most of the cases treatment was reinstituted due to clinical relapse and in some cases on the basis of equal or higher antibody titer at the control exam. Rarely, veterinarians decided to start the treatment again on the basis of skin and lymph node cytology. Control exams were usually performed yearly. Half of the practices lost their patients for follow up and survival time of *Leishmania*-infected dogs was estimated to be 2-5 years.

Half of the practices periodically suggested euthanasia, the rest rarely or never. Nevertheless, 1/3 of *Leishmania*-infected dogs were euthanized anyway.

## *Prevention*

Answers to the prophylactic section were given by 49 out of 105 veterinary practices. Most of them (92,3 %) have advised the use of insecticides and repellents to prevent spread of leishmaniosis. Beside the use of insecticides and repellents, we can see that Slovenian veterinarians have most fre-

**Table 2:** Drugs that have been used by Slovenian veterinary practices in the treatment of dogs with leishmaniosis in the period from 2005 to 2010 (Every number in the table represents answer of one veterinary practice).

| | Never | Periodically | Frequently | Constantly |
|---|---|---|---|---|
| Glucantime (antimoniate) | | 1 | | |
| Pentostam (antimonat) | | | | |
| Pentamidin | | | | |
| Amphotericin B | | | | |
| Miltefosin | | | | |
| Allopurinol | | | | 3 |
| Ketoconazol | | | | 1 |
| Metronidazol | | | | 1 |
| Enrofloxacin | | 1 | | 2 |
| Marbofloxacin | | | | 1 |

**Table 3:** Reasons why Slovenian veterinarians decided for euthanasia of dogs with leishmaniosis (period 2005 to 2010)

| REASON FOR EUTHANASIA | *Frequency* | | | |
|---|---|---|---|---|
| | Never | Periodically | Frequently | Constantly |
| Children in house | 1 | 1 | 2 | |
| Elderly people in the house | 1 | | 1 | |
| People who are receiving immunosuppressive therapy | 1 | | 1 | |
| HIV positive people | 1 | | | |
| Other dogs in the house | | | 1 | |
| Live in an endemic area | 1 | | | |
| Medical expenses | 2 | 1 | | |
| The owner refuses treatment | 1 | 1 | 1 | |
| Kidney failure | 1 | 1 | 1 | 2 |
| Eye lesions | 1 | | | |
| Other causes (specify): -zoonotic risk | | | 1 | |

Comments to table 3: Every number in the table represents answer of one veterinary practice.

quently suggested staying in the house during the night and the use of nets on the windows (Table 4). Seventy-two percent of the practices have advised the use of insecticides and repellents when the dog travelled to an enzootic area even if the owner did not show interest for prevention. Almost half of the practices (48,5 %) instructed the use of permethrin and imidaclopride spot-on combination (Advantix®, Bayer), one third instructed the use of deltametrin collars (Scalibor®, Intervet), and the rest proposed permethrin spot-on (Ex Spot®, Intervet) utilization. One third of the owners (31,3 %) wanted to get instructions on the preventive measures when they were about to move to the endemic area and less of them (22,9 %) if they had already been living in the endemic area.

**Table 4:** Preventive measures suggested from Slovenian veterinarians (period 2005 to 2010) (each number represents answer of one veterinary practice)

| The patient should stay in the house at night | Window nets | Use of repellent insecticides | Use of insect traps (example: electric trap) | Avoiding contact with sick dogs |
|---|---|---|---|---|
| 9 | 9 | 12 | 4 | 5 |

**Table 5:** Owner awareness on the zoonotic potential of leishmaniosis on the basis of the general information (period 2005 to 2010) (each number represents answer of one veterinary practice)

| NEVER | SELDOM | PERIODICALLY | FREQUENTLY | CONSTANTLY |
|---|---|---|---|---|
| 4 | 11 | 1 | 2 | 6 |

**Table 6:** Counsel frequency on zoonotic potential for the owners of dogs with leishmaniosis performed by veterinarians (period 2005 to 2010) (each number represents answer of one veterinary practice)

| NEVER | SELDOM | PERIODICALLY | FREQUENTLY | CONSTANTLY |
|---|---|---|---|---|
| 3 | 5 | 4 | 1 | 13 |

*Zoonotic risk*

Information on zoonotic risk are given in the tables 5 and 6

Majority of dog owners (62,5%) were rarely or never aware about the consequences for their health (Table 5). In contrast, 50 % of Slovenian veterinarians stated to constantly inform the owners of *Leishmania*-infected dogs on consequences for human health, while 46,2 % of them have given information periodically, seldom or never. None of veterinarians encountered a zoonotic case of canine leishmaniosis.

We can see that the majority of dog owners were rarely informed about the consequences for their health on the basis of information given by media during the concerned period.

## Discussion

Due to the global climate changes and enhanced travel of dogs, borders of endemic areas with leishmaniosis in Europe have recently spread towards the north (8, 10). Higher environmental temperatures allow survival of sand flies belonging to the genus Phlebotomus, which are vectors for *L. infantum*. Likewise as the rest of the Europe also Slovenia has been able to observe changes of environmental temperature. Long-term (1961–1990) average summer temperature at Slovenian coast was 21°C. For the last 15 years regular drops below this average level have not been observed any more. That shows the average temperature since 1996 went higher. These data make serious concern about possibility of leishmaniosis spreading. Moreover Slovenia is located between two other Mediterranean countries (Italy and Croatia) where expansion of canine leishmaniosis is a concern.

The global data are based on the experience of 46,7 % of veterinary practices in the country, which makes a very high percentage. However the conclusions can be limited by the relatively low number of veterinary clinics in Slovenia that experienced the diagnosis of the disease.

Amongst the responders, 85,7 % haven't diagnosed a case of leishmaniosis in a dog during the period between 2005 and 2010. All responders shared opinion of not being practicing in enzootic area since they had never seen an autochthonous case. Half of veterinarians believed that number of CanL cases was even getting lower during that period and 50% other believed that prevalence had remained unchanged. Results of this study can be compared to another, done in an enzootic area of southeastern Spain (surface 28000 km$^2$ and 5,5 million inhabitants while surface of Slovenia is 20273 km$^2$ with 2 million inhabitants). In this Spanish survey the percentage of responders was comparable to our survey (47 % and 46,7 %, respectively). Percentages of confirmed Spanish cases ranged from 13 % to 25 % of all dogs examined in veterinary practices during the one-year period, with only 2 % of veterinarians reporting no confirmed cases. Ninety one percent of Spanish responders believed that confirmed CanL cases had become infected within their working area and 58 % of veterinarians believed that local prevalence of leishmaniosis had increased over the preceding ten years and 25% believed that prevalence had remained unchanged (22). In contrast, Slovenian veterinarians observed, that the canine leishmaniosis case numbers are descending or at least stagnating.

It is important for veterinarians to recognize clinical symptoms of leishmaniosis. Most common are skin lesions that are reported in up to 60 % of cases (11, 23). Slovenian veterinarians have constantly to frequently observed skin lesions in CanL, such as alopecia, exfoliative dermatitis, ulcers, nodules, pyoderma, lesions on the bridge of the nose (depigmentation, ulcers) and lesions on footpads (excessive nail growth, hyperkeratosis, ulcers). They have frequently observed eye and eyelid lesions like periocular alopecia, nodules at the edge of the eyelid, conjunctivitis and uveitis. Beside this, non-dermatological signs such as apathy, elevated body temperature, anaemia, weight loss and diarrhea were frequently observed (Table 1). Apathy and weight loss are usually mentioned as consequences of anemia due to lower erythropoiesis associated to the chronic course of leishmaniosis itself or chronic renal failure with lowered erythropoietin production (23). Chronic renal failure is the most common complication of leishmaniosis and also the most common reason for death in dogs (12). Weight loss can be observed in about 25,3 % – 32 % dogs with leishmaniosis (24). Slovenian veterinarians have described weight loss as a constant, very frequent or frequent clinical sign in dogs with leishmaniosis. They have frequently observed signs of chronic renal failure. These results are in accordance to the literature data since histological changes of renal tissue had

been found in 100 % of dogs with leishmaniosis (14). One of the most common clinical sign in CanL is lymphadenopathy which was reported to be present in 65,2 % - 88,7 % of the cases (24). From table 1 it can be seen that Slovenian veterinarians have frequently observed lymph nodes enlargement in dogs with leishmaniosis. Periodically bone and joint changes were observed although leishmaniosis has been rarely described as a cause of lameness in dogs (23). Epistaxis was observed in more than 50 % of Slovenian practices that have diagnosed a CanL case from 2005 to 2010. The result is comparable to surveys in endemic area where 54 % of veterinarians frequently to very frequently observed epistaxis (22). Epistaxis has been described in the literature (5) as a rare but quite suggestive symptom that may be present in about 4 – 10 % of the cases (11).

Amongst serologic tests indirect immunofluorescence antibody test (IFAT) is described as the recommended golden standard method for the diagnosis of clinical cases (25). This method had been used only by one veterinary practice in Slovenia. ELISA test had been used more frequently and that is comparable to results of the study done in Spain (22). ELISA and IFAT are highly sensitive and specific tests for clinically expressed leishmaniosis but are not adapted for confirming asymptomatic dogs (26). Dogs that can express strong cellular immunity are able to control the reproduction of the parasite. Symptoms in these dogs consequently will not appear and they will express no to low antibody titer (low humoral immune response) (27). Slovenian veterinarians had used IDEXX SNAP® Canine Leishmania Antibody Test Kit which is simple for use and is reported to have a high specificity of 90,0-94,7 % and a high sensitivity of 94,7 % (28). Rapid tests are thus useful for diagnosis of sick dogs but do not detect dogs with low antibody levels or asymptomatic dogs. Moreover positive tests are only the first step, as they have to be followed by a quantitative serology for precise evaluation of prognosis and therapy.

Cytology of skin and lymph nodes had been periodically to seldom used by only two Slovenian veterinary practices. In dogs with clinical course of leishmaniosis low to moderate number of parasites can be found (29). Sensitivity of this method can be enhanced by selection of the tissue (the best is skin, popliteal lymph nodes, bone marrow and spleen) (16). Laboratory can enhance the sensitivity of cytological method by expertise or other techniques like immunohistochemistry staining or immunofluorescence on biopsies, to achieve sensitivity from 70 – 100 % (27).

Polymerase chain reaction (PCR) is the most sensitive technique test to detect Leishmania. It uses detection of parasite DNA and may be used on different samples like conjunctival swabs, skin tissue, bone marrow aspirates, lymph node, spleen tissue or blood. Sensitivity of this test usually gets lower with the order of tissues listed above (30). Veterinarians of two Slovenian veterinary practices (28,6 % of all that had diagnosed CanL during the period from 2005 to 2010) have stated to use this method frequently to constantly and they have sent blood samples for PCR, although other tissue then blood would yield to higher sensitivity (30). A positive result by PCR means the presence of the parasite in the sample but not necessary a causative relation with the clinical suspicion and a negative PCR means the DNA was not present in the sample submitted. For these reasons PCR is only a second line diagnostic test after serology to detect the disease. Amongst the different techniques the only recommend are quantitative PCR on kinetoplast DNA (kDNA) (5).

In table 2 drugs that have been used by Slovenian veterinarians in the treatment of dogs with leishmaniosis in the period from 2005 to 2010 are listed. None of nowadays-available medicaments is able to completely eliminate the parasite from the body but can improve clinical state of the dog (31). Meglumine antimoniate (Glucantime ®) and allopurinol are drugs of choice and they should be used in combination. Allopurinol is a "leishmaniostatic" drug and when used alone the percentage of expected relapses after cessation of the treatment is high (32). The possibility to use Allopurinol alone in the treatment of Canine Leishmaniosis is limited to mild forms of the disease (5) which were not the majority of the stages of the disease diagnosed in Slovenia. Meglumin antimoniate is still used rarely in Slovenia because of its high price and inconvenient (parenteral) mode of use. On the contrary, allopurinol is a cheap drug used orally and because of that lots of veterinarians use it alone (12). That was the case also in Slovenian practices in the period from 2005 to 2010. Only one practice had periodically used meglumine antimoniate (Glucantime®). Effectiveness and safety of Miltefosine was recently reported but couldn't have much influence on the treatment protocols in the period from 2005 to 2010 (33).

One third of Slovenian veterinary practices had euthanized their cases in spite of the treatment during the period from 2005 to 2010. This percentage is not surprising taking into consideration that used treatment protocols were not optimal. Dog owners have decided for euthanasia most often because of chronic renal failure (CRF) and sometimes because they have refused the treatment (also because of the cost) and because of the presence of children in the household. Veterinarians have most often elected euthanasia because of CRF (table 3) and zoonotic potential. Recent report showed a lack of evidence that dog culling could diminish visceral leishmaniosis transmission. It was proposed, that animal culling as a control measure of human visceral leishmaniosis should be abandoned (34).

Chronic renal failure is in the literature described as the most common reason for euthanasia (12). In a study performed in France in the period 2002-2004 based on the information obtained from 547 veterinary clinics only 20% of dogs were finally euthanized (8).

High percentage of Slovenian practices that have suggested preventive use of insecticides and repellents (92.3 % of responders) in the period from 2005 to 2010 shows high awareness of Slovenian veterinarians in spite of the fact, that majority of them have never had a case of leishmaniosis. The results of this study are comparable to the study mentioned, that was done in endemic region of Spain, where 92 % of veterinarians suggested use of repellent collars and 74 % suggested spot-on preparations (22). The insecticides that were suggested by Slovenian veterinarians were amongst the most effective (7). Beside chemicals, staying in the house during the night was the most frequently suggested preventive measure by Slovenian veterinarians (18,4 %). Alternative measures (that were mostly experimental in the research period) were not suggested by Slovenian veterinarians opposite to Spanish ones, that have suggested these methods in as high as 25 % although 11 % of them doesn't believe in the efficacy of preventive measures at all (22).

In tables 5 and 6 it can be seen that dog owners were rarely informed on their health consequences from different public media. That can be understood since Slovenia is not known as an endemic region and media very rarely (if at all) publish anything on leishmaniosis. Opposite to general information, a quarter of Slovenian practices (50 % of responders) have constantly informed dog owners on human health consequences.

## Conclusions

The results of this survey, based on the high participation rate of veterinary clinics, show that most veterinarians in Slovenia have good background knowledge on the canine disease and inform the dog owners on the risk for exposed dogs (prevention) or for human beings. This survey also reveals the limits of the diagnostic methods as quantitative serology remains rarely used on suspected cases. Moreover treatment procedure remains frequently limited to the use of allopurinol for both economic and tolerance reasons resulting in a relatively elevated percentage of failure, relapse complications and finally euthanasia.

Most of Slovenian veterinary clinics did not encounter canine leishmaniosis in the period from 2005 to 2010. They all claimed that they do not work in an enzootic or endemic area, since that they did not come across any indigenous cases of this infection. This therefore shows that at least until 2010, Slovenia cannot be considered as an enzootic area. However an increase of travelling dogs to the other Mediterranean countries may result in an increase of the probability of the diagnosis on imported cases. Moreover in a context of very low endemicity the recognition of rare cases can be difficult and only serological or PCR based studies could precise the actual prevalence of the parasite in dogs in Slovenia. We propose for further epidemiological research of canine leishmaniosis in Slovenia, the utilization of diagnostic tests such as IFAT and PCR on a broad population of healthy and diseased dogs, together with vector analysis.

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## LEJŠMANIOZA PRI PSIH V SLOVENIJI, KI JO POVZROČA *LEISHMANIA INFANTUM*: ANALIZA PODATKOV, PRIDOBLJENIH S POMOČJO ANKETIRANJA

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**Povzetek:** Lejšmanioza je bolezen, ki je posledica okužbe z različnimi vrstami protozojskega zajedavca iz rodu *Leishmania*. Od vseh je najbolj razširjena in raziskana okužba psov z vrsto *L. infantum*, ki je nalezljiva tudi za ljudi. Namen naše naloge je bil raziskati stanje lejšmanioze v Sloveniji za preteklo obdobje petih let, od leta 2005 do 2010. Ankete smo poslali 105 klinikam in dobili 49 odgovorov. Izmed tistih, ki so na anketo odgovorili, jih 42 (85,7 %) v zadnjih petih letih pri psih ni diagnosticiralo lejšmanioze. Večina slovenskih veterinarjev meni, da se tendenca novih primerov kot tudi tendenca kontrolnih pregledov psov, obolelih za lejšmaniozo, v obdobju petih let v Sloveniji ni poviševala, temveč je ostajala nespremenjena oziroma je celo upadala. Vsi psi z lejšmaniozo, ki so jih slovenski veterinarji odkrili v preiskovanem obdobju, so bili z že izraženimi kliničnimi znaki pripeljani iz držav z endemično obliko bolezni, večinoma iz Španije in Francije ter občasno iz Portugalske, Italije, Hrvaške in Afrike. Skoraj vedno so slovenski veterinarji opažali kožne spremembe, in sicer brezdlačna mesta, eksfoliativni dermatitis, ulkuse na koži, kožne vozliče, gnojno vnetje kože, spremembe na nosu (depigmentacija, razjede) ter spremembe na blazinicah (hiperkeratoza, razjede). Pogosto so opažali tudi spremembe na očeh in vekah, kot so brezdlačna mesta na vekah, vozlički na robu veke, konjunktivitis in uveitis, pa tudi apatičnost, povišano telesno temperaturo, slabokrvnost, hujšanje in drisko. Tretjino psov z lejšmaniozo so kljub zdravljenju usmrtili, največkrat zaradi odpovedi ledvic in zoonotskega potenciala. Rezultati naše raziskave so pokazali, da Slovenije ni mogoče prištevati med države z endemično obliko bolezni v obdobju do leta 2010. Povečana pogostnost potovanj v mediteranske države pa povečuje verjetnost obolevanja. Za prihodnje študije predlagamo uporabo vektorske analize skupaj z diagnostičnimi testi IFAT in PCR na večjem številu zdravih in obolelih psov.

**Ključne besede:** lejšmanioza; pes; *Leishmania infantum*; zoonoza; peščena muha

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**Article in a journal or newspaper:** Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

**Article in proceedings of a meeting or symposium:** Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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### Načini citiranja

**Knjiga:** Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

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**Članek iz revije ali časopisa:** Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

**Članek iz zbornika referatov:** Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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