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At present rates of growth of agricultural production, biological production, globalization of the transport and external trade relations in the modern world brings to the agenda the issue of food security and biological safety.

The National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', the leading specialized research institution of National Academy of Agrarian Sciences of Ukraine created new 'Journal for veterinary medicine, biotechnology and biosafety'. The first issue included significant data concerning emergent and economical important diseases, biotechnology and genetics in animal breeding and modern biosafety policy issues. The papers were submitted by Ukrainian, Polish and Serbian authors, representing native and foreign veterinary research centers.

The new publishing issue is aimed to promote the life science basic and applied research carried out in Ukraine and other countries. We present the aimed to consolidate and share the new developments and achievements in the aria of biological science. The second volume includes papers devoted to biological safety and veterinary medicine. Journal received International standart serial number (ISSN) for printed and on-line versions.

The Editorial board hopes, that our magazine will be interesting for wide auditorium of scientists and practical specialists in veterinary medicine, biology, biotechnology and biosafety. We invite new authors for fruitful collaboration and joint development of our scientific interactions.

Sincerely yours,
Editors-in-Chief

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GUIDELINES FOR THE PREPARATION OF THE PAPERS SUBMITTED FOR PUBLICATION AT THE 'JOURNAL FOR VETERINARY MEDICINE, BIOTECHNOLOGY AND BIOSAFETY'

1. Papers must be submitted in an electronic variant and should be sent directly to the editorial board at nsc.iecvm.kharkov@gmail.com or inform@vet.kharkov.ua with subject 'Article in JVMBBS'

2. Papers must be written in English

3. Authors make sure there are no typographical errors in the manuscript

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SOFT TICK SAMPLING AND COLLECTION

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Summary. Soft, or argasid, ticks are challenging to sample for or to collect because of their mostly cryptic behaviors that involve crevices, animal burrows, animal nest materials, and digging into soil. Soft ticks generally do not stay attached to their hosts for more than 30 min, hence, examination of living and dead host animals should not be expected to detect specimens in numbers that represent substantial proportions of the total soft tick population in a given area. Sampling provides foundational information that is important for efforts to develop soft tick surveillance programs. Methods applied commonly to sample soft ticks include: manual examination of habitat substrate material, aspiration of host nests or burrows, trapping using CO_2 or possibly other attractants such as pheromones and their analogs. Because African swine fever, caused by a virus, which is highly contagious and afflicts pigs and their close relatives, has been spreading from its usual range in Africa into the Ukraine, we discuss features of the disease and its soft tick (*Ornithodoros* spp.) vectors in order to indicate a contemporary situation involving the need for systematic argasid tick monitoring through sampling.

Keywords: argasid, attractants, collecting, soft tick, surveillance, sampling, African swine fever, trapping, pheromones, *Ornithodoros*

Argasid tick surveillance. There are many important reasons for doing reliable quantitative surveys, and sometimes it is important to determine all of the different tick species in a particular area (qualitative survey), or to determine the range and spread of soft tick species. It is likely that one survey techniques will not be sufficient to conduct an accurate quantitative and qualitative surveys, so a combination of techniques is usually needed. Accurate tick species population estimates are important in evaluating the effectiveness of various control tactics. In the instance of assessing chemical control methods, being able to determine numbers of ticks in their different life stages is essential in both treated and nontreated (control) areas.

Argasid tick distributions can change over time, and these changes are more challenging to predict than those of ixodids. Soft tick modeling is possible and it is based on the natural niche concept, accounting for the influences of climatic factors, nidicolous lifestyle, indiscriminate host feeding, and flexible developmental cycle along diapause periods (Vial, 2009). Accurate knowledge of the distribution of ticks and the monitoring of changes in their distribution are important factors for defining of risk areas for tick-borne diseases and to establish adequate measures for tick control and the prevention of tick-borne disease. For this reason, long-term tick surveillance is a critical component for prevention of widespread and devastating tick-borne disease outbreaks of medical and veterinary significance.

Argasid tick sampling techniques. Each tick species requires optimum environmental conditions and biotypes for its development, which determine their geographic distribution and the pathogens they transmit (Parola and Raoult, 2001). Aspects related to the biology and ecology of argasid ticks, then, need to be taken into consideration to assess their presence in the environment (Uspensky, 2008; Sonenshine and Roe, 2014). These considerations bear relevance in the context of pathogen transmission and the epidemiology of tick-borne diseases (Vial, 2009; Manzano-Román et al., 2012). Certain safety precautions need to be observed when sampling some soft ticks because of their role as vectors of pathogens that affect humans.

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Examination of living or dead host animals. Direct methods for tick surveillance are based on capturing and identifying specimens from vegetation, animal hosts, or other places frequented by ticks. While this works for many ixodid ticks, such methodology is not suited as well for soft ticks. This is mainly because many soft tick species are nidicolous or endophilous, and fast feeders. In such instances, it becomes necessary to investigate all possible soft tick refuges in the sampling area, which is impractical for large-scale studies (Oleaga-Pérez, Pérez-Sánchez, and Encinas-Grandes, 1990; Vial et al., 2006).

In some instances, soft tick collecting is attempted by examining the living or dead bodies of animals that host the target tick species. Animals have been collected from live-animal traps, jaw traps, shooting, road kills, and tranquilizer gun, and by checking game killed by hunters and as road kills (Clymer, Howell, and Hair, 1970; Semtner and Hair, 1973; Tugwell and Lancaster, 1962). Dead animals should be placed inside a container or bag as soon as possible so that ricks detaching from the animals will not be lost. Once the animal has been transported to a laboratory it can be thoroughly examined, and ticks can be removed and preserved for later species identification. It is advised to note the location of the animal body from which the tick was taken (Gladney, 1978). There is a variety of techniques for collecting ticks from wild animals, but special permits are usually required from government agencies, and these administrative requirements should be anticipated and completed before initiating the tick surveys (Gladney, 1978).

Jori et al. (2012) reported a study on the association between *Ornithodoros moubata* (Murray) ticks and warthogs (*Phacochoerus* spp.) and bushpigs (*Potamochoerus* spp.) which was conducted to determine whether wild pig/warthog populations are infected with relapsing fever. Samples were collected from free-ranging animals or by collecting samples from animals killed by hunters (Jori et al., 2012). Collection from free-ranging animals can involve a number of different animal collection techniques followed by immobilizing the animals with a tranquilizer. Small and meso-animals can be live-trapped and checked for ticks (traps can be placed in transects) (Semtner and Hair, 1973; Niebuhr et al., 2013, Labruna et al., 2014), and ticks have been collected from sleeping humans (Labruna et al., 2014). As an example of soft ticks on living hosts, *Argas vespertilionis* (Latreille) was collected manually from trapped Pipistrel bats, *Pipistrellus pipistrellus* (Schreber) (Hosseini-Chegeni and Tavakoli, 2013). Adults and nymphs of *Carios quadridentatus* Heath (Argasidae), a soft tick species associated with the New Zealand lesser short-tailed bat, *Mystacina tuberculata* Gray, become replete in 20–50 min and spend the rest of their lives in guano or crevices in the bat roost, hence, a researcher is much more likely to find larvae than other stages of the tick when bats are examined. Adults and nymphs of fowl ticks, *Argas* spp., are multihost parasites that hide during the say inside chicken houses or near roosting sites, and they engorge rapidly on hosts for only 20–45 min during the night (Diamant and Strickland, 1965). Hence, surveillance for adult and nymph fowl ticks generally requires searching in places where they conceal themselves during the day, but the larvae are commonly found on the host because they attach and feed continuously on one bird for 2–10 d (Gladney, 1978). Surveys for fowl tick larvae can be conducted by examining five birds for attached larvae under wings, on sides of the body, and on the inside of the thighs (Gladney, 1978). Small live-trapped rodents can be removed and placed in wide-mouth plastic jars containing cotton saturated with chloroform. The resulting dead animal can be removed, placed in a plastic freezer bag, labeled, sealed, and stored on ice (Clymer, Howell, and Hair, 1970; Tugwell and Lancaster, 1962). After the animal cadavers cool (detached ticks should be collected and stored), they are placed in a wide-mouthed jar with 50% ethanol which is then shaken vigorously. The animal can be removed from the ethanol and examined carefully for ticks that remain attached. The alcohol is filtered through paper toweling to recover ticks that dropped off while in the jar (Gladney, 1978).

Animals such as raccoons can be anesthetized with ether and released after a visual inspection (Clymer, Howell, and Hair, 1970). Larger animals can be examined by combing over a funnel that leads to a collection jar (Gladney, 1978). With larger animals, blood and mucus often drain out which falls into the funnels. A three-phase strainer is used to separate the bloody mixture from the ticks. The lower screen is 100-mesh screen wire, the second is saran cloth, and the top is 16-mesh screen wire. Ticks are removed by holding the strainer under running water until the blood is washed away (Gladney, 1978). Feral pigs were captured alive using fence traps or by cowboys using a lasso, then the captured pigs were tranquilized before examination for soft ticks (Cançado et al., 2013). Ticks are also collected from road kills, such as *Ornithodoros* spp. being collected manually from coyotes that had been stuck and killed by cars (Bermúdez, González, and García, 2013).

While it might seem intuitive to focus on host animals for collection of soft ticks, argasids usually feed on hosts for only a relatively short time, then drop off to hide in crevices, soil, and nesting material. Animal traps should be checked at least twice each day because ticks begin to leave a dead body after a few hours (Gladney, 1978). Some species, including species of *Ornithodoros*, feed nocturnally for as little as 10 min and are not found on hosts during daylight hours, further complicating efforts to collect them on the bodies of animals (Butler and Gibbs, 1984). In order to more accurately assess tick populations or to maximize specimen captures, using techniques to collect from such abiotic habitats is recommended (Gladney, 1978; Manzano-Román et al., 2012).

Examining soil, debris, and other substrates. *Ornithodoros coniceps* (Canestrini) is an ornithophilic species and its life cycle is strictly connected with the pigeon (wild and domestic). It when not feeding, it inhabits cracks and crevices where pigeons nest. These places include ancient towers, bell towers, old houses, ruins, ledges, and attics (Khoury et al., 2011). Manual collection of *O. coniceps* in one study consisted of picking up visible ticks moving on the wall using forceps (Khoury et al., 2011). Similarly, the bat tick, *O. mimon* Kohls, Clifford et Jones, was collected from internal walls of human dwellings, beds, ceilings, and attics (Labruna et al., 2014). *Ornothodoros rostratus* Aragão and *O. brasiliensis* Aragão, known colloquially at 'ground ticks' because both species live buried in sand or soft land near host habitats, mainly parasitize rodents, pigs, *Conepatus* sp. (skunk), Tayassus sp. (javelina) (Martins et al., 2011). Sampling for ground ticks has been conducted by examining cellars, stables, and primitive human habitations (Martins et al., 2011). Adult *O. guaporensis* Nava were collected manually from a rocky fissure inhabited by bats in the Amazonian forest, Bolivia (Nava et al., 2013).

Soil, debris, nesting material, and other nonhost substrates in which soft ticks hide can also be examined manually using forceps (Latif and Walker, 2004). The material can be examined where it was found, or it can be collected and examined later (Niebuhr et al., 2013). Sampling for *O. erraticus* in Portugal was accomplished by removing the dust and contents of crevices and holes in stone walled pens and the wooden or tiled roofs over a white cloth, which was examined for soft ticks (Caiado et al., 1990). The pavement of the pig sties was also dug out, especially around the walls and in the resting places of the pigs, and the soil was examined for soft ticks (Caiado et al., 1990). *Ornithodoros capensis* Neumann has been found in the nest material of brown pelicans, *Pelecanus occidentalis* L., and the ticks, which cause nest desertion by the pelicans, are readily obtained by visual examination of pelican nest material and manually removing the ticks from it (Keirans, Hutcheson, and Oliver, 1992). Similarly, *Argas arboreus* Kaiser, Hoogstraal et Kohls was manually collected from nests of cattle egrets, *Bubulcus ibis* (L.) (Mumcuoglu et al., 2005).

A debris-filtering method was developed to filter topsoil, bedding material, and other debris of varying size and composition to collect adult *O. megnini* ticks found within and around locations frequented by ungulates and other hosts (Niebuhr et al., 2013). The method involved three screens with hole sizes of 1.3 × 1.3 cm, 0.6 × 0.6 cm, and 0.3 × 0.3 cm affixed within wooden frames and stacked in order of hole size (largest on top) to serve as the filter apparatus (Niebuhr et al., 2013). Soil sieving was also used to collect adult *O. rostratus* and *O. brasiliensis* (Martins et al., 2011). In a mark-recapture study on *Argas reflexus* (F.), which parasitizes pigeons, *Columba livia* (Gmelin), the ticks were trapped in house attics using smooth V-shaped metal gutters attached to the attic walls (Dautel et al., 1994).

A major drawback to examining soil, debris, and other substrates for soft ticks is that it can be laborious and time consuming. Also, because of the small size of larval and nymphal stages, and their dark coloration which makes them difficult to see, individuals can go undetected in the sample substrate. Therefore, examination of soil and debris is not a desirable sampling method for large-scale studies.

Vacuuming. Trapping ticks inhabiting animal burrows can be challenging. Nocturnal burrow dwelling ticks are secretive, hiding or digging into the soil when disturbed (Uspensky 2008; Anderson and Magnarelli, 2008). Their presence on a host is limited to periods of feeding, which may be completed in as little as ten minutes. After taking a blood meal, they rapidly return to their hiding place. The use of vacuum sampling devices has facilitated surveying tick distribution in the burrow habitat (Butler et al., 1984). Vacuum devices allow the removal of live ticks from the burrow habitat in significant numbers when other survey methods are unable to determine their presence (Butler et al., 1985). For collecting *Ornithodoros* spp. near animal burrows and swine holding areas, a vacuum system was used employing a modified ECHO power blower (model 202) mounted in an aluminum case on an internal door which housed a nylon collection bag (Butler et al., 1985). The vacuum produced by the blower pulled air from the burrow through a 3-m-long crushproof vacuum hose 4 cm in diameter (Butler et al., 1985). Specimen collections of *O. coniceps* have been performed by various methods, including battery-operated aspirators that permitted collection of ticks along corners and cracks that could not be reached manually (Khoury et al., 2011). In a study that involved searching for *Ornithodoros sonrai* Sautet et Witkowski on pigeons in Senegalese villages, a portable gasoline-powered vacuum cleaner adapted for burrow-dwelling ticks was used (Vial and Martins, 1984; Vial et al., 2007).

Advantages of vacuum collecting include ease of finding fuel for running the vacuum, the equipment is relatively inexpensive, a fuel-powered vacuum can be run autonomously which is conducive to long field missions, the suction tube can probe and collect ticks from deep fissures and burrows, all stages of the tick are captured, and it is known where the ticks were immediately before being captured. Disadvantages include the possibility of the operator being bitten by ticks and being exposed to fuel fumes (wear protective clothing to avoid both), and although vacuuming is more rapid for collecting ticks than manual examination of substrates, it is slower than collecting by using CO_2 as an attractant (no empirical comparison, however, has been reported for vacuum collecting versus manual and CO_2 collecting.

Traps and attractants. Carbon dioxide (CO_2) emanating from the host is a chemoattractant for *Ornithodoros* ticks (Garcia, 1962; Nevill, 1964; Khoury et al., 2011). Dry ice has been used as a source of CO_2 in devices used to lure *Ornithodoros* ticks for different purposes (Adeyeye and Butler, 1991; Vredevoe n. d.). Larvae of *O. coriaceus* were collected with a CO_2 trap while none could be obtained by the continual handpicking method (Hokama and Howarth, 1977). Schwan et al. (2009) used a white terry cloth towel wrapped around small blacks of dry ice to attract ticks. Each trap was taped to the end of a 1-m-long stick and placed in recesses of a room. One collection trap involves using compressed CO_2 gas in a small (e. g., 2.27-kg) cylinder (Niebuhr et al., 2013). Two pieces of 8-m-long clear vinyl tubing with a 0.635-cm diameter attached to a brass Y-valve connected to a gas flow regulator on the cylinder. Using a 22-gauge needle, two holes were made at opposite sides of the tubing in 1-m increments, and each end was sealed with a metal eye-bolt (which allowed for each end of the tubing to be staked to the ground or hung if desired) (Niebuhr et al., 2013). Once a trap was set, 16 'sample squares' of white fabric per trap were placed over each pair of perforations to allow ticks to attach and subsequently be collected. The sample squares were comprised of white 200-thread count cotton fabric affixed to metal frames (0.33 × 0.33 m). In one study the CO_2 cylinder was opened for 30 min with a flow rate of CO_2 from the regulator into the tubing at ≈ 0.28 m^3/h during each use (Niebuhr et al., 2013).

The CO_2 collection method allowed to evaluate the involvement of *O. erraticus* in the maintenance and transmission of African swine fever virus (ASFV) in Portugal (Caiado et al., 1990). Fifty-gram chunks of dry ice placed at various distances from tick-infested gopher tortoise burrows used to assess *Ornithodoros turicata* (Dugès) responses at various distances from tick-infested gopher tortoise burrows collected ticks up to eight meters away from the burrows in 2 hours (Adeyeye and Butler, 1991). Differences in attraction were not detected in a 1-h period using 500 to 2,000 ml CO_2/min (Adeyeye and Butler, 1991). Higher numbers of *O. coniceps* nymphs, and adult males and females were collected with a CO_2 trap as compared to employing an aspirator (Khoury et al., 2011). It must be noted, however, that factors such as season, ambient temperature, tick population dynamics, and host availability influence how many, and the life stage of specimens attracted

by CO_2. A convenient and effective CO_2 soft tick trapping device was a stainless-steel tray (30 × 45 × 8 cm) carrying a polystyrene plastic (Styrofoam) cup of ~ 500 ml capacity which was filled with solid CO_2 pellets (Caiado et al., 1990). The traps were placed in the ground with soil or other bedding material covering them to their top edges and left for 1–24 h depending on the severity of local infestation, season, and ambient temperature (activity of soft ticks decreases at temperatures < 15 °C), and the eventual presence of vertebrate hosts using the premised for the night (Caiado et al., 1990).

Advantages to making CO_2 collections are that the traps can be left unattended, and the equipment is easy to handle and assemble, the materials used in the apparatus is inexpensive, the exposure of research personnel to tick bites is low, large number of ticks can be collected regardless of their life stage, and the method simulated vertebrate hosts by exuding CO_2. Disadvantages of using CO_2 to collect soft ticks include scarcity of dry ice in some places, dry ice storage at room temperature is relatively short (3 d), hence, it is not conducive for long periods in the field. The method is also less effective in deep burrows (e. g., warthogs) than in shallower burrows, and the precise place where the soft ticks were residing is not determinable. Despite the disadvantages of the CO_2 collection method, a study conducted in pig sties by Caiado et al. (1990) showed that the success rate was 70% in contrast to only 10% when sampling was restricted to manual means.

Other attractants to soft ticks have been tested. A mixture of guanine hydrochloride and diatomaceous earth in saline was used as an attractant in bioassays, causing 53.1–95.7% assembly, and the attractant was mixed with acaricides to reduce their repellency and enhance their efficiency in bioassays (Gothe, Week, and Kraiss, 1984; Dusbábek et al., 1997). A modification of a synthetic analog of an assembly pheromone was used as follows: a mixture of 5 mg of guanine hydrochloride and 5 mg of diatomaceous earth as pheromone carrier (1:1 w/w) was dispersed in 220 µl of 0.85% NaCl solution (Dusbábek, Jegorov, and Šimek, 1991). In the instance of the soft tick *Argas walkerae* Kaiser et Hoogstraal an assembly pheromone was used to attract ticks to filter paper discs impregnated with the pyrethroid flumethrin (Gothe, Week, and Kraiss, 1984).

Serology. The challenges to soft tick surveillance indicate a need for serological tests (e. g., ELISA) as an indirect method. Such serological methods detect specific antibodies against tick salivary proteins in serum samples taken from hosts. Development of this approach requires resolution of several factors, including 1) the host species to be sampled (domestic animals are preferred if available), 2) demonstration that the tick species induces a humoral immune response, 3) characterization of the response in terms of the number of tick bites needed to induce detectable antibody levels, and how long antibodies remain at detectable levels after the last tick bite, and 4) which antigen should be used and it sensitivity and specificity (Manzano-Román et al., 2012). Serological tests have been developed for *O. erraticus* in southern Europe and for *O. moubata* in Africa. Such tests could help to identify vectoring tick species populations that can be targeted for control, possibly eliminating the diseases that the tick species transmits. While tick salivary gland extract for the two *Ornithodoros* species are suitable antigens for serological surveillance, the method has some drawbacks that include being difficult to standardize, time-consuming collection, poorly known composition, and possible inclusion of nonspecific antigens that could result in confounding cross-reactivity.

Other sampling methods. Additionally, techniques utilizing the natural fluorescence of ticks when exposed to ultraviolet light allow observations on nocturnal behavior to be made (Butler and Gibbs, 1984; Latif and Walker, 2004).

African swine fever and its *Ornithodoros* spp. tick vectors. Several soft tick species in the genus *Ornithodoros* are vectors of ASFV in nature, or known to be susceptible to infection (Kleiboeker and Scoles, 2001). African swine fever (ASF) caused by ASFV is considered one of the most serious transboundary swine diseases because of its high lethality for pigs, its crippling socio-economic consequences, its propensity for rapid and unanticipated international spread, and the absence of either treatment or vaccine (FAO, 2009). Presently the *Ornithodoros* is moving into the Ukraine, the northern range limit of *Ornithodoros* spp. in the Palearctic region being 47°N (Filippova, 1966). However, there is no surveillance for soft ticks and the pathogens they transmit in the Ukraine. The Ukraine is surrounded by territory in which ASFV is present and therefore has legitimate concern for the introduction of ASF. Recent developments in Eastern Europe indicate that further geographic expansion of ASF is likely to occur, requiring increased prevention and vigilance to protect swine populations and the associated business and livelihoods (FAO, 2012).

In the United States, ASF is considered a high-consequence foreign animal disease. As such, ASF is classified in the first of three tiers with other diseases because it poses a significant threat to animal agriculture at the national level by having the highest risks and consequences (APHIS, 2013). Native soft tick species and the exploding feral swine population pose risks for the emergence of ASF in the United States. Some soft tick species native to the United States. have been shown to be competent ASFV vectors in the laboratory. Wild pigs, like the wild boar, that are native to Europe and feral hogs that are abundant in the United States represent a potential reservoir population for the virus (Jori and Bastos, 2009), which is a risk for the emergence of ASF in new parts of the world. Establishment of an endemic infection in these regions would make eradication difficult or impossible. An ecological approach is required to evaluate the potential for temporal and spatial interactions between soft ticks and feral swine, which may provide a pathway for spillover of ASF into the United States if the disease emerged in the southern transboundary region.

The upsurge of ASF in many areas of the world has the potential to cause a continuing panzootic crisis. The Ukraine shares borders with the Russian Federation and Georgia, both of which are experiencing ASF epizootics. By initiating a surveillance program in wild pigs and soft ticks, the Ukraine will be able to enhance its veterinary services infrastructure by developing a detection system for the emergence of ASF, and the development of an emergency response that will maximize the chances of eradicating outbreaks if they were to occur. Similar systems would be applicable to the United States and other countries with feral swine populations.

It is considered that because of their long life (up to 15 years) and strong resistance to starvation and persistence of infection for at least 5 years, ticks of the *O. erraticus* (Lucas) complex can be important in maintaining local foci of ASFV, which can lead to endemicity, in regions encompassing Trans Caucasian Countries and the Russian Federation (EFSA …, 2010a; Boinas et al., 2011). *Ornithodoros* ticks can feed on pigs, from which the vectors can be infected. The epidemiological role played by soft ticks becomes

important where pigs are managed under traditional systems, including old shelters/sties with crevices.

The European wild boar (*Sus scrofa* L.) is distributed throughout the Ukraine. Wild boars are as susceptible to ASFV infection as domestic pigs. Wild boar populations are a risk for the introduction of ASF to the European Union (Blome, Gabriel, and Beer, 2013; De la Torre et al., 2013). In Eastern Europe the current distribution and density of *Ornithodoros* ticks, whether feeding on pigs or wild boar, and their ability to maintain ASFV or transmit the virus to suids remain largely unknown. There is an urgent need for more research in those areas (FAO, 2013).

There are approximately 36 species of *Ornithodoros* ticks in the world that show these general characteristics: i) nidicolous lifestyle, ii) indiscriminate host feeding and short bloodmeal duration, and iii) flexible developmental cycles via diapause periods (Uspensky, 2008; Vial, 2009). Methods applied commonly to sample these ticks include: handpicking, aspiration of host nests or burrows, baiting and trapping using CO_2. Although handpicking ticks in their natural habitat may be considered a crude surveying method, in some cases this is the only practical approach to encounter some soft tick species (Robert, 2002). Continued research on the application of serological methods for surveillance will help monitor tick occurrence and their involvement in the epidemiology of ASFV (Ravaomanana et al., 2011). There have been several reports of surveillance of *Ornithodoros* spp. vectors conducted among domestic pig populations in Portugal and Spain (Boinas et al., 2011; Caiado et al., 1990; Oleaga-Pérez, Pérez-Sánchez, and Encinas-Grandes, 1990; Pérez-Sánchez et al., 1994) and in the ASF-endemic/epidemic regions of Africa among both domestic pig populations and warthogs (Ravaomanana et al., 2010; Vial et al., 2007; Haresnape, Lungu, and Mamu, 1987). Although *Ornithodoros* tick have been reported in the Caucasus region, their current species composition and distribution remains to be fully understood (EFSA …, 2010a; Diaz et al., 2012). The *Ornithodoros* species present in the Trans Caucasus Countries and the Russian Federation reportedly fall within the *O. erraticus* group and their vector capacity and ability for ASFV remain to be tested (EFSA …, 2010b).

The global spread of ASF is a concern for the United States. The four species of *Ornithodoros* soft ticks from North America and the Caribbean Basin that have been experimentally infected with ASF virus (ASFV) are: *O. coriaceus* Koch; *O. turicata*; *O. parkeri* Cooley and *O. puertoricensis* Fox (Hess et al., 1987). ASF outbreaks have occurred in Central and South America, and the Caribbean. *O. turicata* is considered a potential vector of ASFV in north central Florida (Butler and Gibbs, 1984).

The term 'feral hog' tends to be used generically to refer to Eurasian wild boars, domesticated hogs that have become feral, and their hybrid offspring. Feral pigs, used here as a synonym for feral hogs, have been shown to be highly susceptible to experimental infection with ASFV. The problem is such at the Texas-Mexico border that Mexican officials planned to cull 50,000 feral hogs from the United States invading Mexico and affecting 3,700 acres of farmland. Life history traits of feral hogs and soft ticks facilitate their ecological interaction in time and space. Feral pigs are burrowing animals. Soft ticks tend to live in the burrows of their primary hosts. In Florida, *O. turicata* fed readily on caged piglets that were placed at the aprons of burrows inhabited by the ticks and gopher tortoises (Adeyeye and Butler, 1989). Preliminary serological data indicates infection with *B. turicatae*, which suggests that feral swine in Texas are exposed to *O. turicata*, which is the biological vector of that causing agent of human tick relapsing fever (Sanders, 2011). The infestation with soft ticks of feral pigs crossing the border between Mexico and the United States in the ecosystem comprising south Texas provides a potential pathway for the introduction of ASFV into the national herd of hogs and pigs that as of March 2012 included 64.9 million head, which is vital to an industry yielding an expected total of 23.3 billion pounds of pig meat in 2012. Texas' national ranking in terms of total number of pigs produced hovers between 15th and 18th. Over 48,000 hogs are raised annually for show.

We tested the hypothesis that feral pigs may share the same habitat with *O. turicata* by looking for the ticks in area where they were found to infest other hosts like Neotoma rats. Using CO_2 as bait, we attracted and collected *O. turicata* from a rodent nest that was likely inhabited by Neotoma rats in the northern part of the Rio Grande Plains ecological area in Texas. It must be noted that *Ornithodoros* ticks have been found to infest feral swine in Brazil (Cançado et al., 2013).

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EPIDEMIOLOGY OF BOVINE SPONGIFORM ENCEPHALOPATHY IN CATTLE IN POLAND

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Summary. The aim of the paper was to present the epidemiological situation regarding BSE epidemic in Poland with respect to cattle population, active surveillance and the control measures. Epidemiological data from the archives of the national reference laboratory for animal TSEs at the National Veterinary Research Institute and from the archives of the General Veterinary Inspectorate in Warsaw were used in the study. Between 2001 and the end of April 2015 BSE was diagnosed in 75 animals. Sixty one cases were classical BSE and 14 were atypical BSE (12 of L-type and 2 of H-type). Almost 6 million animals were tested using rapid tests. Dynamics of C-type BSE shows constant rise until 2005 when the highest number of cases (20) was recorded with sharp drop in the following years. Prevalence of atypical BSE shows stable trend with slight fluctuations. Traditional feeding was used in 65 and 90% of classical and atypical BSE cases, respectively. On the other hand, traditional feed was supplemented with MBM and milk replacers in 46 and 10% of classical and atypical BSE cases, respectively. Despite the high infectious load introduced into Poland especially with MBM, the number of cases was relatively low. In Poland, relatively high number of atypical BSE cases was recorded, comprising 19% of all BSE-positive animals. Mean age of classical BSE cases diagnosed annually does not show a decreasing trend which may reflect the late introduction of feed ban in Poland.

Keywords: bovine spongiform encephalopathy, Poland, prevalence, prion protein, control measures

Introduction. The outbreak of bovine spongiform encephalopathy (BSE) in cattle followed by the discovery of variant Creutzfeldt-Jakob disease (vCJD) linked with the consumption of BSE-contaminated food raised major concerns about human health.

Experience from UK and other European countries indicated that the use of contaminated meat-and-bone meal (MBM) in animal rations was the main route of BSE spread in cattle. However, the origin of BSE is still elusive and under discussion. Many countries denied any BSE risk in their native born cattle until they found first cases. Active surveillance with rapid tests carried out at the abattoir and in the fallen stock improved strongly the detection of BSE, and the first case was diagnosed in Poland in May 2002. From 2002 through 2015 (end of April), 75 cases were diagnosed including classical cases and atypical cases. Main control measures adopted in Poland to monitor the disease and minimize the risk of spreading of the agent included: passive surveillance of clinical suspects (introduced in 1996), MBM feed ban for ruminants (1999), active surveillance using rapid tests in healthy slaughtered and risk group cattle (January 2001), mandatory removal of specified risk material (SRM) from animals intended for human consumption (April 2001), proper treatment of animal waste (introduced in 2002) and feed ban of processed animal protein for all farmed animals (2003).

The aim of the paper is to present the epidemiological situation regarding BSE epidemic in Poland with respect to cattle population, active surveillance and the control measures.

Material and methods. Epidemiological data from the archives of the National Reference Laboratory for Animal Transmissible Spongiform Encephalopathies (TSEs) at the National Veterinary Research Institute in Pulawy and from the archives of the General Veterinary Inspectorate in Warsaw were used in the study. Additionally, information provided in annual reports from Member States on BSE and scrapie published by the Directorate-General for Health and Consumer Protection of the European Commission were utilized.

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Results. ***Implementation schedule of active surveillance and mass testing to detect BSE***. Poland started testing for BSE in 1996 when clinical suspects were subjected to histopathological examination. While this target group proved to have the highest prevalence rate of BSE (positive cases per 10 000 animals tested), the number of samples submitted annually for testing was very low (up to 844 samples per year). Active surveillance using rapid tests was initiated in January 2001 and became fully operational in November 2001, when 4 regional labs started routine testing. Over 110 000 samples were tested before the first case was diagnosed and confirmed in May 2002. Since then the number of samples tested per year has been growing from 286 592 in 2002 to 637 130 samples in 2010 (Tab. 1). A high percentage of samples tested came from healthy slaughtered animals; it varied between 97% of all samples tested in 2002 and 90.5% in 2007, along with the constant increase of the number of samples from fallen stock submitted to BSE testing (from 0.7% in 2002 to 7.9% in 2007). The full implementation of testing regime in terms of the number of samples required to be tested began in mid 2002.

Epidemiological situation regarding BSE. Between 2001 and the end of April 2015 BSE was diagnosed in 75 animals of which 57 were healthy slaughtered (76% of all cases), 5 were emergency slaughtered, 9 were fallen stock, 3 cases were found as clinical suspects and 1 case was a cohort animal. All BSE cases were diagnosed in native born cattle. Sixty one cases were identified as classical BSE and 14 – atypical BSE (12 of L-type and 2 of H-type). Almost 6 million animals were tested using rapid tests, which means that the detection of one case, on average, required the testing of 79 670 animals. The distribution of cases and animals tested per year is presented in Table 1.

Dynamics of C-type BSE shows constant rise until 2005 when the highest number of cases (20) was recorded with sharp drop in the following years. Prevalence of atypical BSE shows stable trend with slight fluctuations (Fig. 1). Except for 2006 and 2008 when single H-type cases were found all other atypical cases were of L-type.

Table – 1 Healthy slaughtered and risk group animals (fallen stock, emergency slaughter, clinical suspect, sanitary slaughter) tested with rapid tests for BSE in years 2001–2013 and the number of confirmed cases of classical and atypical BSE (until the end of April 2015)

Testing period	Healthy slaughter	Risk group	Total tested	Classical BSE	Atypical BSE
2001.11–12	29 882	427	30 309	0	0
2002	278 709	7 883	286 592	3	1
2003	428 452	26 961	455 413	5	0
2004	445 198	35 918	481 116	9	2
2005	472 028	43 948	515 976	18	2
2006	540 148	53 973	594 121	8	2
2007	546 243	57 567	603 810	7	2
2008	556 583	54 983	611 566	5	0
2009	587 339	50 733	638 072	3	1
2010	590 171	47 069	637 240	2	0
2011	440 856	35 050	475 906	0	1
2012	299 682	26 598	326 280	1	2
2013	280 145	38 704	318 849	0	1
Total	**5 495 436**	**479 814**	**5 975 250**	**61**	**14**

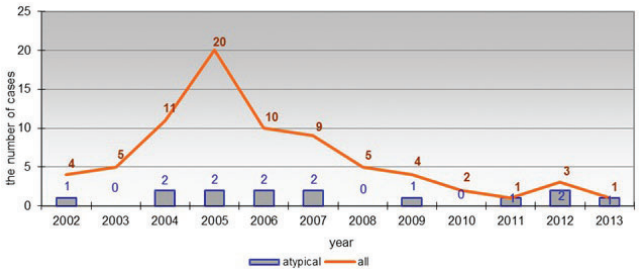

Figure 1. The number of all BSE cases and atypical BSE cases diagnosed in Poland in years 2002–2013

The annual incidence rate of BSE (defined as a number of cases per 1 million of animals tested annually) is shown in Figure 2.

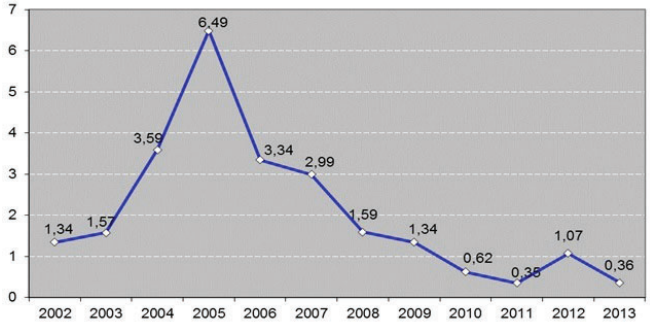

Figure 2. Annual incidence rate of BSE cases in Poland in years 2002–2013

Since atypical BSE is found only in older animals (8 years and above), age structure of cattle population could be a possible explanation for the relatively high frequency of the appearance of this form of BSE in Poland. On average animals of 7 years and above comprised 41% of all cattle tested in years 2002–2010. Mean age of classical and atypical BSE cases was 8 and 12 years, respectively.

Mean age of classical BSE cases can be an indicator that the disease is fading away when the average age of BSE cases identified in consecutive years is constantly growing. Such a trend is directly related to the successful implementation and the enforcement of control measures like feed ban or SRM removal. Growing age of positive cases was observed from 2009 until 2011 (Fig. 3). Then in 2012 a classical BSE case was diagnosed in 6 years old cow and since then no classical BSE cases were identified in Poland. Such an unstable trend indicates that the time span from the introduction of control measures (total feed ban was introduced in November 2003) to the time when their effect is observed is too short and it requires further surveillance.

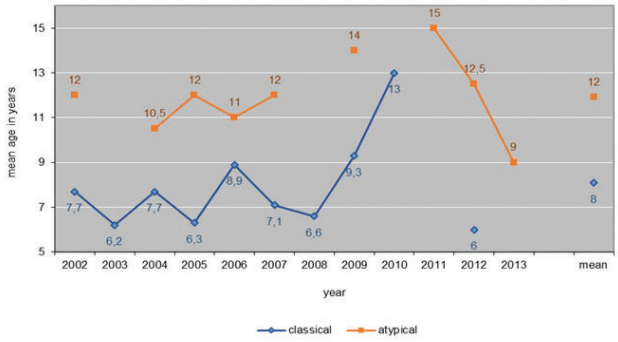

Figure 3. Mean age of positive cases with respect to classical and atypical BSE in years 2002–2013

Feeding data is available for 62 cases (52 classical BSE and 10 atypicals). Traditional feeding was used in 65 and 90% of classical and atypical BSE cases, respectively. On the other hand, traditional feed was supplemented with MBM and milk replacers in 46 and 10% of classical and atypical BSE cases, respectively.

Discussion. BSE epidemic in Poland can be analyzed from various perspectives. One of the parameters can be the size of the epidemic with respect to the infectious load introduced to Poland with live animals and meat-and-bone meal imported from BSE affected countries. Despite the high infectious load introduced into Poland especially with MBM, the number of cases was relatively low reaching 75 cases by the end of April 2015.

The number of samples tested for BSE reached almost 6 million samples meaning that the detection of a single case required the testing of almost 80 000 animals.

Another important aspect is the internal stability meaning the power to control the disease and stopping its spread by implementing various protective measures. If we consider a constant age distribution of the population over time during the period of interest, the main simple indicator of a decreasing trend of the BSE epidemic in a given country is an increasing of the mean age of the cases found in the years following the introduction of control measures. However, since the incubation time of BSE is 4–6 years on average, it takes several years before any conclusion can be drawn. Poland introduced the ruminant feed ban in 1999 and the total feed ban in 2003 meaning that between 2003–2005 and 2008–2010 the decline in the number of positive cases and increasing mean age

should be observed. For Poland, this holds true only for the annual incidence rate, reaching its peak with 20 cases in 2005, followed by sharp drop in the following years. Opposite to this observation, the mean age of positive cases fluctuated from year to year from 2002 to 2008; however, the increase of the mean age of classical BSE cases in 2009 and 2011, though based on a very few cases, is encouraging but needs confirmation in the following years. In addition to the drop in the number of cases detected in the past years, it might be the sign of the declining of the epidemic, and could be linked to the implementation of the total feed ban in 2003. However, the very recent classical BSE case was diagnosed in a 6 years old cow born in 2006.

Another interesting observation is the relatively high number of atypical BSE cases recorded among positive cases comprising 18.6% of all BSE-positive animals. The prevalence rate of atypical BSE, which was 2.1 cases per million tested animals, whatever their age, was more than twice to the value observed in France (0.76 as the sum of 0.41 for H-type and 0.35 for L-type). Of all atypical cases, 64% was found in healthy slaughtered animals, while for classical BSE this value was 80%. Feeding data gives additional proof that atypical BSE could be a spontaneous form of this disease not related to MBM feeding.

Conclusions. Despite significant amount of MBM imported to Poland, the number of BSE cases diagnosed is rather low, at 75 cases. More than 18% of all BSE cases were classified as atypical BSE, regarded as the spontaneous form of BSE not related to feeding habits. Last case was diagnosed in Poland in February 2013, and it was atypical BSE. Mean age of classical BSE cases diagnosed annually does not show a decreasing trend which may reflect the late introduction of feed ban in Poland.

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INFLUENCE OF PHYTONUTRIENT 'VITASTIM' ON CHICKEN MUCOSAL IMMUNITY AFTER INFECTION WITH LOW-PATHOLOGICAL AVIAN INFLUENZA VIRUS

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Summary. The aim of our study was to investigate the immunostimulatory effect of phytonutrient 'Vitastim' on the immune response after avian infection with low pathogenic avian influenza virus.

It was conducted three groups of experimental chickens of 6 week old (21 birds in each group) to study the mucosal immunity of chickens after experimental infection with low-pathological avian influenza virus, and application of phytonutrient 'Vitastim'. Cryostat sections of 7-μm thickness were prepared, and detection of cells was done using unlabeled primary monoclonal antibodies against the CD4, CD8, IgM, IgG, IgA, macrophage antigens, and a commercially available staining kit. As a negative control, slides were incubated with PBS instead of the monoclonal antibodies. Sections were counterstained with hematoxylin and mounted with Canada balsam. Statistical analysis was performed using SPSS 17.0 software for Windows.

By the results of immunohistochemical research of influence of immunostimulating phytogenous preparation 'Vitastim' on organism of chickens there was determined that given preparation more actively influence on humoral cell of immune reaction in norm and at lowly pathogenic avian influenza (caeca, trachea, lungs) that testified more intensive formation and accumulation of B-lymphocytes which produce immunoglobulins. At research of spleen, there was determined amplified proliferation of T-lymphocytes, macrophages that characterize the activation of cell immune reaction.

The results of immunohistochemical studies of inner organs of chickens of 3 research groups, it was established the influence of the phytonutrient 'Vitastim' on humoral immune system, as evidenced by high levels of IgM, IgG, IgA. 'Vitastim' had stimulating impact on state of cell immunity, however, as evidenced by the low levels of CD4, macrophages, in chickens of experimental groups compared to the intact birds, cell immunity does not play a significant role in the pathogenesis of LPAI.

Keywords: avian influenza virus, cell-, and humoral-mediated immune response, immunohistochemistry, low pathogenic avian influenza

Introduction. Poultry production now is rapidly developing and needs of modern veterinary support. The presence of accelerated evolutionary processes resulted in a complication of the epizootic situation, increasing the pathogenic properties of the pathogens, the spread of infectious diseases.

Way out lies in enhancing the natural resistance of the organism, strengthen the immune status at the level of the organism and group immunity by eliminating the immunosuppressive factors and use immunostimulatory means. Immunostimulation is widely used in infectious disease. There is used the adjuvants of different origin. They are valuable way to improve the immune status of the avian organism and enhance the immune response during vaccination. Search for new immunostimulating preparations continues to view ever-increasing requirements regarding their safety, effectiveness, and accessibility.

Recently, considerable attention is paid to herbal preparations (Lee et al., 2008, 2011). Many plants are well known in ethnoscience and have pronounced immunostimulatory properties. *Echinacea purpurea* has such properties. The root alcoholic extract is prepared from this plant (Kogut and Klasing, 2009).

Herbal immunostimulants also prepared by combining several plants in view of the focus of their actions on the organism. Such immunostimulant significantly increases the level of antibodies in vaccinated birds (Lee et al., 2010). Phytonutrient 'Vitastim' showed a high immunostimulating activity and harmlessness at using for chickens under experimental conditions (Krasnikov et al., 2002).

Materials and methods. *Experimental animals.* It was used 63 6 week-old chickens to study mucosal immunity state after application of phytonutrient 'Vitastim'. It was formed 3 groups from them:

1. Chickens experimentally infected with law pathogenic avian influenza virus A/mallard/Ukraine/2007 H5N2 intraintranasally in the dose 10^3 EID_{50} per chicken, and given phytonutritin 'Vitastim' in the doze of 2 mg/kg;

2. Chickens, which were given phytonutritin 'Vitastim' in the doze of 2 mg/kg;

3. Control untreated chickens.

Water and feed were provided *ad libitum*.

Immunohistochemistry. Pathological material was collected on 1st, 3rd, 5th, 7th, 10th, 14th, and 21st days post infection. Than it was prepared histological sections from spleen, caeca, trachea, lung fixed in liquid nitrogene. Cryostat sections of 7-μm thickness were prepared, and detection of cells was done using unlabeled primary monoclonal antibodies against the CD4, CD8, IgM, IgG, IgA, macrophage antigens, (Southern Biotechnology Associates, Eching, Germany) and a commercially available staining kit (LSAB, ChemMate Detection kit, peroxidase antiperoxidase, rabbit/mouse; DakoCytomation, Hamburg, Germany). As a negative control, slides were incubated with PBS instead of the monoclonal antibodies. Sections were counterstained with hematoxylin and mounted with Canadian balsam (Riedel de Haen AG, Seelze-Hannover, Germany).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software for Windows. All data for each group expressed as means ± SEM. The difference between the means considered at $p < 0.05$.

Results. *Immunohistochemistry.* With the purpose of immunohistochemical research of forming of immune answer of chickens' monoclonal antibodies were applied to subpopulations of immunocompetent cells: CD4, CD8, IgM, IgG, IgA, macrophages and M-cells.

At the detailed study of dynamics of forming of immune answer in birds infected by the virus of lowly pathogenic influenza + phytonutrient 'Vitastim' (the 1st group), in intact birds, which got the phytonutrient 'Vitastim' (the 2nd group), and control birds (the 3rd group) the next changes of amount of immunocompetent cells were detected in inner organs.

Therefore, in a spleen at investigation of cells CD4 observed the increase of these cells already on the 3rd day of experiment (26.930 ± 0.767%) against 18.193 ± 4.602% — on the 1st day of experiments. Process of increase lasted to 7th day, when the amount of cells acquired maximal values (22.153 ± 0.378% against 18.213 ± 0.431% — in a control bird). Maximal indexes in chickens of the 1st experimental group the amount of cells after period of suppression attained on 10th day (21.03 ± 0.421% at 18.213 ± 0.751% — in control). Moreover, the level of CD4 on 10th–21st days of experiments was higher than in chickens of the 2nd and the 3rd groups. There has been noticed that in an intact bird the percent amount of cells-helpers decreased since 7th day (20.943 ± 2.047%) and to the end of term of observation on 21st day (13.056 ± 0.246%). The fact of increase of subpopulation of cells CD4 in the chickens of the 2nd group on a background the reduction of amount in a control bird testifies immunostimulating influence of preparation 'Vitastim' (Fig. 1).

At the study of subpopulation of lymphocytes with the superficial marker of CD8 a considerable increase of the percent of these cells in experimental and control birds. As in the case with CD4, the amount of CD8 began to increase on 3rd day, thus its amount in experimental birds was far above, than in control. So, on 3rd day in chickens of the 2nd group the amount of CD8 was 37.246 ± 0.763% against 21.503 ± 3.579% — in a control bird. Starting with 5th day the percent amount of CD8 began to decrease. However in the experimental bird of the 2nd group during two weeks after the task of preparation observed a trend to the increase of amount of these cells with a maximal index on 10th day — 28.933 ± 4.065% against 26.380 ± 0.575% — in an intact bird. Maximal indexes in chickens of the 1st experimental group the amount of cells attained on the 7th day with index 29.136 ± 0.604% at 28.836 ± 0.759% — in control. The level of CD8 in chickens of the 1st group on 10th–21st days of experiments was higher than in chickens of the 2nd and the 3rd experimental groups.

Content of macrophages in a spleen was characterized by a reliable increase from first day of observation (8.936 ± 1.843% on 3rd day against 7.146 ± 1.023% on 1st day of investigations in chickens of the 2nd group. The level of macrophages differed low difference between the experimental and control bird but it was higher in chickens of the 1st group with the maximal index on the 7th day 11.463 ± 0.419. Process of reliable increase of amount of this subpopulation of cells in chickens of the 2nd group lasted to 7th day and attained to 11.443 ± 0.399% against 10.153 ± 0.580% in the group of control. The percent amount of macrophages decreased in future but was higher than on 1st day of investigations (7.033 ± 0.431% in the 1st group against 6.378 ± 1.452 — in intact chickens.

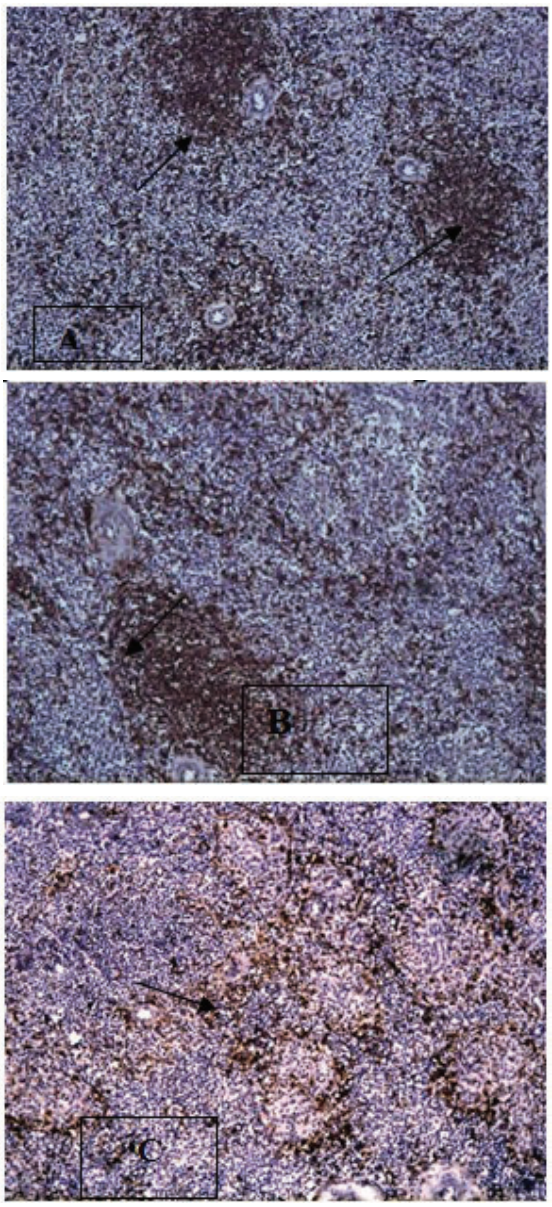

Figure 1. CD4 — aggregates in chicken spleen on 5th day after infection with LPAIV + phytonutrient 'Vitastim' (A), phytonutrient 'Vitastim' feeding (B), and in control chickens (C). Arrows show on cell aggregates in the form of conglomerates and dots of brown colour. It is also shown that the number of cells in the spleen of chickens of 1st and 2nd groups (A, B) prevail over the control (C). LSAB-method, × 200.

An amount of IgM-expressing cells in the first three days of observation was considerably greater in chickens of the 2nd group, than in the 1st group and control. Therefore, on 1st day their percent amount presented 3.566 ± 0.405% against 3.45 ± 0.900% in the control group. In addition, the process of reliable increase of amount of these cells was fixed in a spleen with a maximal index on 10th day, which in chickens of the 2nd group presented 3.653 ± 1.888% at 2.010 ± 1.168% in the group of intact chickens. In chickens of the

1st experimental group this index was higher than in chickens of the 2nd and 3rd groups and presented 4.193 ± 0.433%. Than the percent of these cells began to decrease but was considerably greater in the group of infected chickens, which got 'Vitastim'

At the immunohistochemical study of content of IgG-expressing cells in the histological sections of spleen was not carried out the tendency to the gradual increase or decrease of percent amount of these cells, only oscillation in ether, one or another side. But starting with 10th day of observations we are carried out the increase of amount of cells of this subpopulation in chickens of the 2nd group from 6.240 ± 2.124% at 3.710 ± 1.211% — in the control group that lasted to the end of term of observations on 21st day, when the amount of these cells got the highest value (8.546 ± 0.452% at 5.951 ± 0.521% — in the group of intact chickens. But from 10th day the amount of cells in chickens of the 1st group begun considerably increase and was higher than in the 2nd and in the 3rd groups to the end of term of observations attaining the maximal index 9.07 ± 0.191%. The process of the percent of cells IgG was with decrease of IgM percent. Possibly, it is connected with change of protective functional from IgM to IgG (Fig. 2, 3).

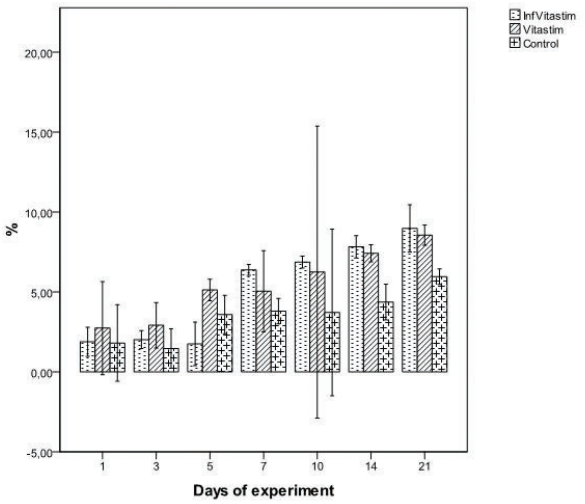

Figure 2. Dynamics of IgG changes in chicken spleen after phytonutrient 'Vitastim' feeding. Each bar represents the accumulation of cells in the immune process dynamics since 1st to 21st days after phytonutrient administration. Significant stimulating influence of phytonutrient 'Vitastim' on immune response against LPAI is shown since 7th day. Statistical analysis was performed by comparing cell amount in spleen of chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim), phytonutrient 'Vitastim' feeding (Vitastim) with control birds.

At the investigation of caeca there was determined gradual increase of cells with superficial marker CD4 from 1st day (4.826 ± 0.337% at 2.950 ± 0.265% in control) to 5th day (6.756 ± 0.241% at 4.32 ± 0.536% in (intact) chickens of the 2nd group). But in chickens of the 1st group after low suppression on 3rd–5th days was determined the increase of amount of cells on 10th day with maximal index 24.456 ± 2.249%. Moreover, the level of cells in chickens of the 1st group was far above during all period of observations. After that in chickens of the 2nd group starting with 7th day the process of activation of immune system became sharp character when the indexes on 7th and 14th days were 9.823 ± 1.289% and 14.726 ± 0.534%. The amount of cells-helpers in chickens of the 2nd group significantly overstated the amount in control group in this interval (5.980 ± 0.420% and 7.090 ± 0.725% on 7th and 14th days respectively). On 21st day the percent content CD4 decreased but significantly overstated the indexes on 1st day of investigations.

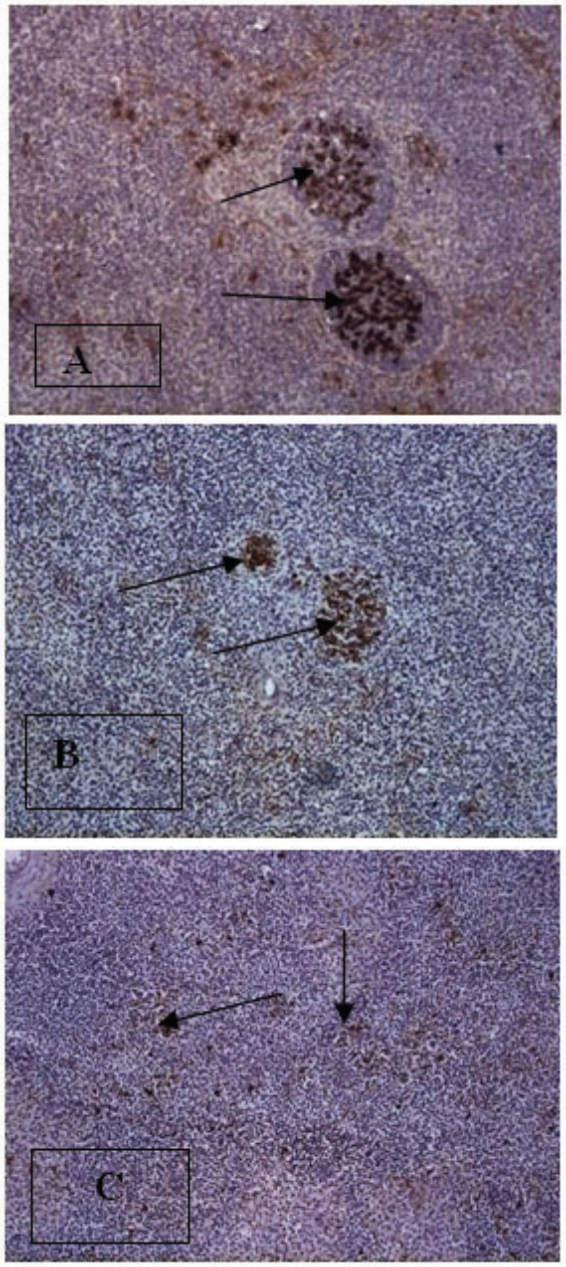

Figure 3. IgG aggregates in chicken spleen on 14th day after infection with LPAIV + phytonutrient 'Vitastim' (A), phytonutrient 'Vitastim' feeding (B), and in control chickens (C). Arrows show on cell aggregates in the form of conglomerates and dots of brown colour. It is shown an immunostimulative influence of phytonutrient 'Vitastim' that proved by intensive accumulation of IgG in germinative lymphoid follicles (A, B) as compared with control (C). LSAB-method, × 200.

The dynamics of accumulation of cells with superficial marker CD8 in chickens of the 2nd group was characterized by the insignificant, gradual increase of percent amount from 1st (11.716 ± 2.855%

at 4.853 ± 1.834% in the group of control chickens) to 10th day inclusive (15.356 ± 3.933% at 10.763 ± 1.168% in control). Maximal values the percent of cells attained on 14th day — 19.833 ± 0.245% at 12.143 ± 0.149% in intact chickens, after that there was an insignificant decrease of activity of these cells, which however was higher than in the previous terms of research. As for chickens of the 1st group during the period of suppression from 1st to 5th days the level of these cells was far above than in other groups of chickens. So on 1st day the level of CD8 in chickens of the 2nd group presented 20.28 ± 0.384% 4.853 ± 1.834% in the group of control chickens. On 14th day the level of cells begun decreased and the indexes were lowest than the level of chickens of the 2nd and the 3rd groups.

Chickens which got preparation 'Vitastim' (the 2nd group) characterized by getting up of functional activity from 3rd day (1.866 ± 0.190% at 2.343 ± 0.669% in control) to 10th day inclusive with a maximal index 4.063 ± 0.193% at 2.833 ± 0.162% in intact birds have an amount of IgM-expressing cells. In further observed the period of slump of activity to the end of term of observations on 21st (2.178 ± 0.184% at 1.896 ± 0.254% in the group of control). In chickens of the 1st experimental group the indexes during all period of research were less than in chickens of the 2nd and the 3rd groups with maximal level on 2.226 ± 0.585% — on 10th day of observations.

These data correlated with data of changes of the content of IgG-expressing cells. So, in a period, when observed the rise of activity of IgM (that is from 1st to 10th days of research), the level of IgG hesitated towards to the increase and decrease of activity. However, on 10th day, when the percent amount of IgM decreased, there was an activation of making of IgG. So, on 10th day of observation in chickens of the 2nd group its content presented 6.910 ± 0.026% at 3.113 ± 0.239% in control chickens. The process of functional activity rise of these cells in course of time acquired sharp character and arrived at maximal values on 21st day of experiment (7.012 ± 0.237% at 3.894 ± 0.219% in the 2nd group of intact chickens. But in chickens of the 1st group the process of increase of percent amount attained more sharp character than in chickens of the 2nd and the 3rd groups with maximal index 9.983 ± 0.128% on 21st day of observations.

At research of dynamics of IgA-expressing cells did not observe a clear dynamics to the increase or decrease of percent amount of these cells. Moreover, the level of IgA in chickens of the 2nd group did not almost differ from indexes of control chickens (3.960 ± 0.310% at 3.486 ± 0.244% in intact bird on 5th day of research). Only beginning from 14th day of observations in chickens of the 2nd group the clear increase of content of cells was specify with a marker to 4.533 ± 0.538% at 3.826 ± 0.615% in control that lasted to the end of term of observations on 21st day and presented 6.146 ± 0.394% at 5.124 ± 0.407% — in an intact bird. However, as in previous case the level of cells in chickens of the 1st group during 7th–21st days was considerable higher than in chickens of the 2nd and the 3rd groups with maximal index 12.923 ± 0.334% on 21st day of studying (Fig. 4).

Such reliable increase of IgA can testify about positive influence of applied immunostimulating phytonutrient 'Vitastim' on the state of local immunity which takes place on the mucous membranes of respiratory and gastrointestinal tracts.

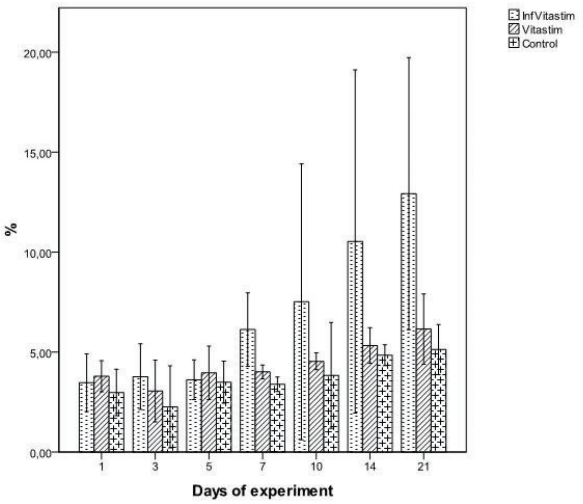

Figure 4. Dynamics of IgA changes in chicken caeca after after phytonutrient 'Vitastim' feeding. Each bar represents the accumulation of cells in the immune process dynamics since 1st to 21st days after phytonutrient administration. Significant stimulating influence of phytonutrient 'Vitastim' on immune response against LPAI (especially in chickens infected with LPAIV + phytonutrient 'Vitastim') is shown since 7th day. Statistical analysis was performed by comparing cell amount in caeca of chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim), phytonutrient 'Vitastim' feeding (Vitastim) with control birds.

During CD4 examination in lungs the sharp increase of their activity was established almost in two times in the 1st group of chickens which got preparation 'Vitastim'. Therefore, on 3rd day the number of CD4 was 4.170 ± 0.205% at 2.416 ± 0.335% in control. However, on 7th day in the group of control there was observed the sharp increase of cells to 4.026 ± 0.123%. Thus, phytonutrient 'Vitastim' stimulates activation of process of accumulation of CD8 earlier. Stimulation of immune answer took place in the chickens of the 2nd group to 14th day of study, when the content of killers acquired maximal numbers and was 5.920 ± 0.703% against 3.676 ± 0.182% in control. As for the group of intact chickens, then a tendency to the gradual decrease of activity of CD4 was observed already on 10th day (3.930 ± 0.472%) and lasted to the end of term of observations on 21st day. However in chickens of the 1st experimental group after period of suppression on 1st–5th days of experiments observed the period of sharp rise of activity of these cells on 7th day, moreover their amount on 7th–10th days was far above than in chickens of the 2nd and the 3rd groups and presented 6.683 ± 0.576% on 10th day. After that was observed percent decrease of these cells but their amount was higher than in control.

CD8 had similar dynamics with CD4. Therefore, the insignificant increase of cells in relation to control was observed in chickens of the 2nd group from the 3rd day (3.963 ± 0.977% at 2.976 ± 0.261% in the group of control) and lasted to 5th day with an index 4.863 ± 0.933% at 4.436 ± 0.449% in the group of intact birds. However, from 5th day the process of increase of cell activity acquired sharp character and presented 7.410 ± 0.266% at 5.243 ± 0.222% in the chickens of control group. The maximal reliable indexes of CD8 were in the chickens of the 2nd group on 10th day (8.276 ± 0.250% at 5.080 ± 0.540% — in the group of intact chickens). On 14th day and to the end of term of observations occurred very slow decrease of the

percent account of CD8 as opposed to the control chickens, where the activity decrease was significant on 21st day (3.214 ± 0.813% at 5.173 ± 0.200 — on 14th day of research). Dynamics of changes of CD4 was almost similar in chickens of the 1st experimental group. During the first ten days of observations, their amount was higher than in chickens of the 2nd and the 3rd groups The subpopulation has maximal indexes on 10th day — 3.88 ± 0.576%. After that, there was observed the percent decrease of these cells but their amount was higher than in control.

Indexes of macrophages in lungs were considerably less, than in spleen, but the character of accumulation almost not differed (Fig. 5).

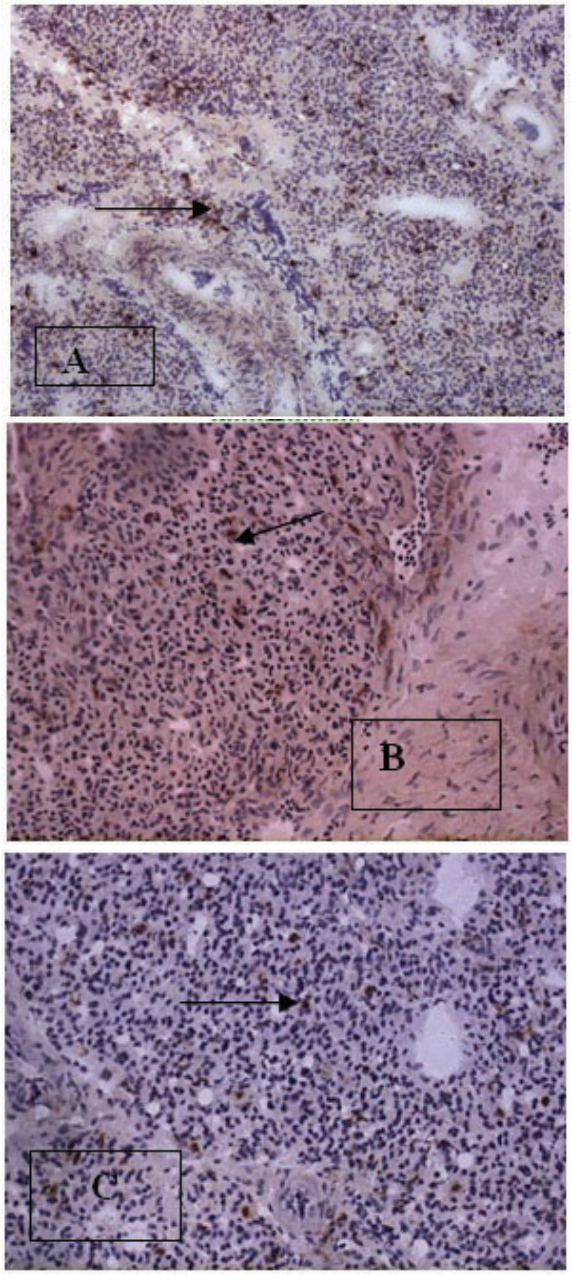

Figure. 5. Macrophage aggregates in chicken lung on 3rd day after infection with LPAIV + phytonutrient 'Vitastim' (A), phytonutrient 'Vitastim' feeding (B), and in control chickens (C). Arrows show on cell aggregates in the form of brown coloured dots. It is shown small ammount of these cells in the lung of chickens of all experimental groups during period of observation. LSAB-method, × 200.

Therefore, since first time of observations it was determined the tendency to the gradual increase of percent amount of macrophages, as in the group of chickens, which got phytonutrient 'Vitastim', and in control, intact birds. But if in control group sometimes insignificant oscillation of amount of cells has been observed toward decrease or increase then in chickens of the 2nd experimental group the process of activation of accumulation of macrophages had a clear tendency to the increase to 5th day of research, when the level of cells acquired maximal index 6.793 ± 0.473% and 5.663 ± 0.342% (in chickens of the 1st and the 2nd groups) at 3.7 ± 0.365% in intact birds. After that from 7th day the insignificant slump of activity of macrophages, that lasted to the end of term of observations on 21st day and presented 4.296 ± 0.467% and 4.264 ± 0.452% (in chickens of the 1st and the 2nd groups) at 3.347 ± 0.512% — in control.

At research of IgG-expressing cells is determined its insignificant quantity in the first five days of observations. However, on 7th day in chickens of the 2nd group was a sharp insignificant increase of the percent of their cells on a background of sharp decrease of IgM-expressing cells (5.653 ± 0.139% at 4.113 ± 0.133% in an intact bird) but their level in control group was considerably below during 7th–21st days after treatment (6.532 ± 0.235% at 4.540 ± 0.214% in control at the end of study). A sharp increase of IgG-expressing cells in chickens of the 1st group occurred on 10th day (4.18 ± 0.407% at 4.046 ± 0.278% — in control). In future there was observed the percent increase of these cells in chickens of the 1st group with maximal index 5.643 ± 0.238% on 21st day of research, moreover their level was higher than control during all period of observations.

The vibrations of percent amount of cells in the side of increase or decrease observed at the study of subpopulation of IgA-expressing cells. A clear tendency to the increase to the percent of cells was marked from 10th day of research (3.946 ± 0.315% and 3.023 ± 0.800% in chickens of 1st and 2nd groups at 1.846 ± 0.290% in intact chickens). The increase of activity of cells lasted to the end of term of observations on 21st day and acquired a maximal level with an index 6.063 ± 0.239% and 5.214 ± 0.124% at 2.412 ± 0.135% in the group of control chickens. Moreover during all period of observations the amount of cells in chickens of 1st group was greater than in chickens of 2nd and 3rd groups (Fig. 6).

In trachea when studying CD4 watched for an insignificant trend to the increase of percent amount of these cells in the chickens of the 2nd group during the first five days. So, on 5th day the content of CD4 presented 1.516 ± 0.318% against 0.883 ± 0.141% in the control group of chickens. Starting with 7th day there was a considerable increase (almost in two times) of cells of this subpopulation, attaining the maximal indexes on 10th day (2.743 ± 0.254% — in chickens of the 2nd group at 1.876 ± 0.812% in an intact bird). In further there was a process of rapid slump of activity of these cells, which lasted to the end of term of observations on 21st day (1.856 ± 0.121% — in chickens of the 2nd group at 0.964 ± 0.136% in the chickens of control group). As for chickens of the 1st group that curve of changes of accumulation dynamics characterized by considerable amount of cells concerning to chickens of the 2nd and the 3rd groups during all period of observations (almost in three times greater). So on 1st day the amount of cells presented 3.41 ± 0.617% at 1.033 ± 0.224% and 0.526 ± 0.255% — in chickens of the 2nd and the 3rd groups. Cells with markers CD4 attained the maximal index on 10th day (4.686 ± 0.282%). On 14th–21st days of observations in chickens of the 1st group occurred the process of slow decrease of cell amount.

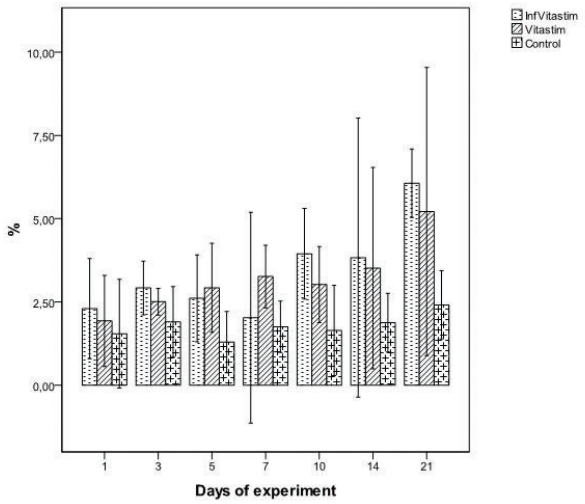

Figure 6. Dynamics of IgA changes in chicken lungs after phytonutrient 'Vitastim' feeding. Each bar represents the accumulation of cells in the immune process dynamics since 1st to 21st days after phytonutrient administration. Significant stimulating influence of phytonutrient 'Vitastim' on immune response against LPAI is shown since 3rd day. Statistical analysis was performed by comparing cell amount in lung of chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim), phytonutrient 'Vitastim' feeding (Vitastim) with control birds.

At the study, CD8 it is registered its significant amount in chickens of the 1st group comparatively with the 2nd group and the group of control more than in three times during all period of study. During all time there was a clear tendency to the increase of percent amount of these cells. Maximal indexes of CD8 attained on the 10th day of research, when its level presented in chickens of the 1st group — 5.13 ± 0.461%, in the 2nd group of chickens 2.216 ± 0.708% at 1.166 ± 0.156 in the group of control bird. In a further period of research observed the insignificant decrease of amount of these cells to the end of term of experiments on the 21st day (Fig. 7).

When studying IgG in chickens of the 2nd group is determined its insignificant amount in the first seven days of observations (0.396 ± 0.044% at 0.196 ± 0.042% in the bird of control group. Exactly on 10th day a sharp reliable increase of percent of their cells was marked on a background of the decrease of IgM-expressing cells (1.770 ± 0.183% at 0.343 ± 0.117% — in intact chickens. An increase lasted to ends of term of observations on 21st day, when an index acquired maximal values — 6.542 ± 0.162% at 0.984 ± 0416% — in the control group of chickens. In chickens of the 1st group there was also determined insignificant its amount in first seven days of observations (0.573 ± 0.078%). but on 10th day the amount of cells considerably grew and exceeded levels in chickens of the 2nd and the 3rd groups with maximal index on 21st day 3.72 ± 0.190% (Fig. 8).

A clear tendency to the IgA increasing to the percent of cells was marked from 10th day of research (2.09 ± 0.175% and 1.640 ± 0.160% in chickens of the 1st and the 2nd groups at 1.076 ± 0.218% in control bird) and to the end of term of observations with a maximal level on 21st day — 2.876 ± 0.518% and 2.312 ± 0.185% in chickens of the 1st and the 2nd groups at 1.554 ± 0.156% in group of intact chickens. As in previous case the amount of cells in birds of the 1st group was far above than in chickens of the 2nd and the 3rd groups.

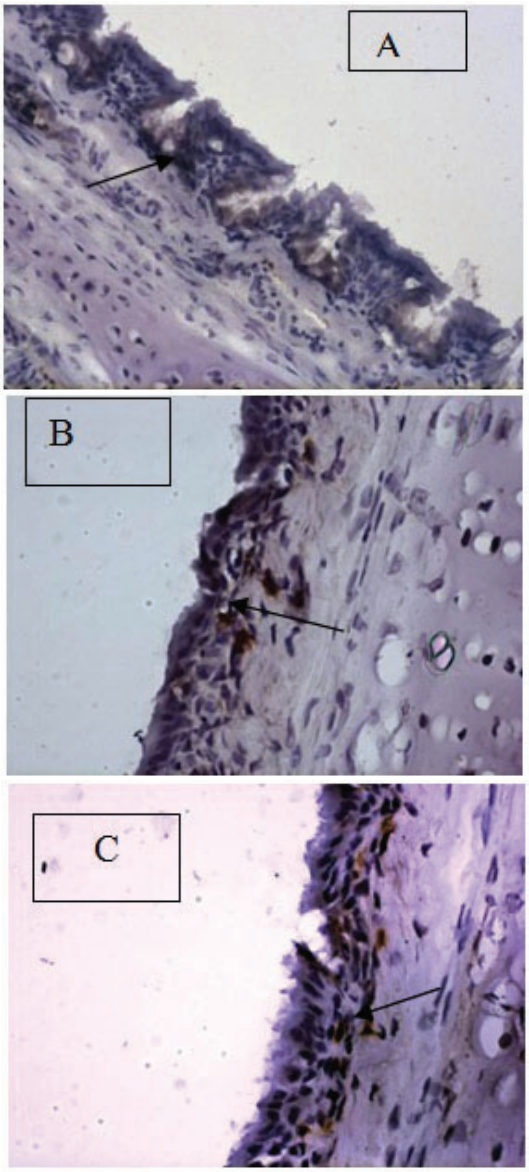

Figure 7. CD7 aggregates in chicken trachea on 3rd day after infection with LPAIV + phytonutrient 'Vitastim' (A), phytonutrient 'Vitastim' feeding (B), and in control chickens (C). Arrows show on cell aggregates in the form of brown coloured dots. It is shown small ammount of these cells in the traches of chickens of all experimental groups during period of observation. LSAB-method, × 200.

Conclusion. Data of realized research certify that character of cell amount changes of different subpopulations in a spleen, lungs, caeca and trachea of chickens of 1st, 2nd and 3rd groups is not differed substantially. However, there are existed some differences.

Differences testified that in a spleen (comparatively with first day of research) on 1st–10th days took place sharp increase of amount of CD8, macrophages. On 10th–14th days there was an increase of amount of IgG and IgA at some diminishing of content of cells with the markers of macrophages and IgG comparatively with 7th day of research. On 14th–21st days the certain stabilization of content of clusters was marked at level, near to their value on 10th day.

Content of IgA-expressing cells notedly grew short in a period between 1st and 7th days and rose in future, arriving at a maximum on 21st day.

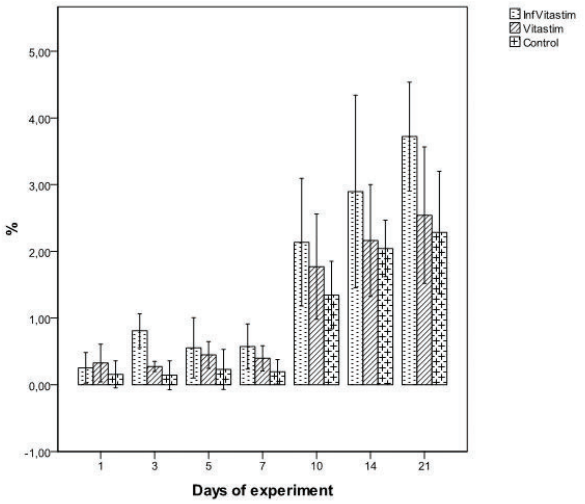

Figure 8. Dynamics of IgG changes in chicken trachea after phytonutrient 'Vitastim' feeding. Each bar represents the accumulation of cells in the immune process dynamics since 1st to 21st days after phytonutrient administration. Significant stimulating influence of phytonutrient 'Vitastim' on immune response against LPAI (especially in chickens infected with LPAIV + phytonutrient 'Vitastim') is shown since 10th day. Statistical analysis was performed by comparing cell amount in lung of chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim), phytonutrient 'Vitastim' feeding (Vitastim) with control birds.

In lungs paid attention on itself higher indexes of content of IgG and IgA (almost in two times) in chickens of group 1 and 2 in relation to chickens of control group, beginning from 10th day. At the same time on 10th day observed sharp diminishing of IgM-expressing cells, which lasted to the end of term of observation on 21st day what testifies about the transmission of function of protection from IgM to IgG on it stage of immune process. At research of content of CD4 and CD8 observed a trend to the increase of these cells of 7th for 14th days of research at decrease of them in intact chickens, that can testify about immunostimulating influence of preparation 'Vitastim' on the immune state of experimental bird.

In a caeca observed the sharp increase of CD4, since 10th day especially in chickens of 1st group in relation to control chickens. The process of increase lasted to (the end of term of observations) 10th day. Amount composition of these cells prevailed over the amount of CD4 in lungs that can testify about more considerable role of this organ in forming of immune reaction. Moreover, the amount of macrophages in all investigational organs considerably grew already in the first terms of research that testified that they get the first in the process of immune reaction.

In the trachea of intact chickens, which got preparation 'Vitastim' and chickens of 1st group observed the considerable predominating of cells with markers of CD8, IgM above the level of these cells in a control bird. On the end of term of observations looked after the maximal level of IgG, which was greater than control bird in two times. Immunohistochemical determination of the amount of cells with the marker IgG can be the criterion of determination of activity of immunostimulating preparations. There were less CD4 than in other investigational organs during all period of observations.

By the results of immunohistochemical research of influence of immunostimulating phytonutrient 'Vitastim' on organism of chickens there was determined that given preparation more actively influence on humoral cell of immune reaction in norm and at lowly pathogenic avian influenza (caeca, trachea, lungs) that testified more intensive formation and accumulation of B-lymphocytes which produce immunoglobulins. At research of spleen, there was determined amplified proliferation of T-lymphocytes, macrophages that characterize the activation of cell immune reaction.

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Part 2. Veterinary medicine

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SEROEPIDEMIOLOGICAL RESEARCH OF BABESIOSIS IN DOGS IN THE AREA OF NOVI SAD, AUTONOMOUS PROVINCE OF VOJVODINA, REPUBLIC OF SERBIA

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Summary. Canine babesiosis is emerging tick-borne disease of dogs. Due to the expansion of the vector, increase in the incidence of this disease in Europe is evident. The aim of this research is to conduct a seroepidemiological cross-sectional study and to establish the value of seroprevalence of canine babesiosis caused by *B. canis*.

The study included population of dogs from the area of Novi Sad. Blood sera from 191 dogs were serologically tested using indirect immunofluorescent antibody test. The dogs were divided into three groups as following: hunting dogs, companion dogs and stray dogs.

The determined overall seroprevalence of *B. canis* infection in the examined dog population was 26.17%. The highest seroprevalence of this infection was evident in the group of stray dogs (35.0%), followed by seroprevalence — in the group of hunting dogs (32.75%), and the lowest — in the group of companion dogs (13.7%). Divergence in the results obtained in our research and previous studies conducted in Serbia indicates the necessity of more extensive seroepidemiological and molecular studies of canine babesiosis.

Keywords: canine babesiosis, *Babesia canis,* seroprevalence, dogs, indirect immunofluorescent antibody test, Serbia.

Introduction. Canine babesiosis is one of the most important transmitted by ticks infectious diseases of dogs. Although known since 1893, this disease remains a challenge for diagnosis and treatment to the veterinarians worldwide (Penzhorn, 2011).

In Europe the predominant causative agent of canine babesiosis is *Babesia canis*, videlicet its two subspecies: *B. c. canis* transmitted by *Dermacentor reticulatus* ticks and *B. c. vogeli* transmitted by *Rhipicephalus sanguineus* (Hamel et al., 2009; Matijatko et al., 2014). The disease has enzootic character and occurs seasonally in spring and summer when ticks are most active (Bourdoiseau, 2006). The clinical presentation of canine babesiosis is diverse and implies a combination of a febrile syndrome and a hemolytic syndrome, which can be fatal when complicated (Matijatko, Torti and Schetters, 2012; Solano-Gallego and Baneth, 2011).

Recently canine babesiosis, the disease considered to be endemic in Southern Europe, has been reported as an autochthonous in several more northern European countries such as Holland, Latvia, and Norway (Berzina et al., 2013; Matjila et al., 2005; Øines, Storli and Brun-Hansen, 2010). With significant variations amongst countries and regions, caused by the vector distribution, Halos et al. (2014) have determined the overall annual incidence of 0.7% of clinical babesiosis in the investigated dog population.

For quite some time presence of *B. canis* has been confirmed in Serbia both in several tick species (*R. sanguineus, D. marginatus, D. reticulatus,* and *H. conncina*) and in dogs. Determination of babesiosis in dogs was based mostly on microscopic examination of blood smears, still Potkonjak et al. (2014) recently confirmed the presence of specific antibodies to the *B. canis* antigens in hunting dog sera using IFA test (Mihaljica et al., 2012; Tomanović et al., 2013; Pavlović et al., 2009).

* Corresponding author

The aim of this research is to conduct a seroepidemiological cross-sectional study and to establish the value of seroprevalence of canine babesiosis caused by *B. canis*.

Materials and methods. The study included population of dogs from the area of Novi Sad. Blood sera from 191 dogs were tested. The dogs were divided into three groups as following: hunting dogs, companion dogs and stray dogs. From the samples of the full venous blood, after the retraction of blood clot in vacationers with coagulation activator, samples of blood serum were separated by centrifugation at 3 000 rpm for 10 min and frozen at – 20 °C till the indirect immunofluorescence was carried out.

Immunofluorescent antibody test 'MegaScreen® FLUOBABESIA canis' manufactured by 'MEGACOR Diagnostik' (Austria) was used for the serological investigation. Concerning specific geographical area, only the titer of 1:128 was used, and all reactions positive for this and higher titers were declared as positive. Positive reaction was characterized by the appearance of sharply defined apple-green fluorescent merozoites within the cytoplasm of the infected erythrocytes.

Results. Anti-Babesia canis antibodies of class G were detected using indirect immunofluorescent antibody test in 50 samples from the 191 examined dog blood sera. The determined overall seroprevalence of infection caused by *B. canis* in examined dog populations was 26.17% (Fig. 1).

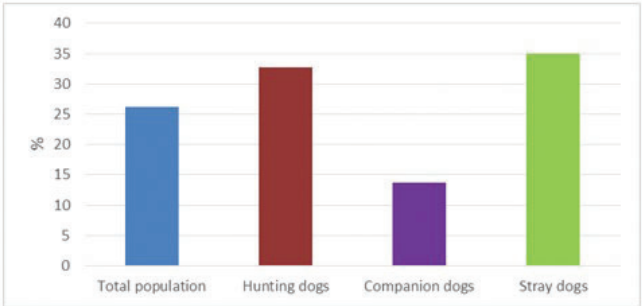

Figure 1. Comparative review of the determined values of seroprevalence of canine babesiosis among different dog groups.

In the examined population of 58 hunting dogs seropositive were 19 dogs. Seroprevalence of *B. canis* infection determined in this group was 32.75% (Fig. 1).

In the group of companion dogs 10 from the 73 examined dogs were seropositive. Seroprevalence of *B. canis* infection determined in this group was 13.7% (Fig. 1).

In the group of stray dogs from the 60 examined dogs specific antibodies to *B. canis* antigens were found in sera of 21 dogs. Seroprevalence of *B. canis* infection determined in this group was 35.0% (Fig. 1).

Discussion and conclusions. The determined overall seroprevalence of *B. canis* infection was 26.17% in the examined dog population. The highest seroprevalence of this infection was evident in the group of stray dogs (35.0%), followed by seroprevalence — in the group of hunting dogs (32.75%), and the lowest — in the group of companion dogs (13.7%).

In accordance with the increase in the number of the vectors and the proven presence of *B. canis* both in the vectors and in dogs, serological confirmation of the pathogen in dogs was expected (Potkonjak et al., 2014). During the period 1999–2001 in the area of Belgrade, by microscopic examination of stained blood smears, Pavlović et al. (2002) determined the prevalence values of *B. canis* infection among dogs ranging from 71,7 to 75,5%. These seroprevalence values were significantly higher than the value obtained in this research. In addition, very high seroprevalence of *B. canis* in dogs has been reported in Italy (70%) (Pennisi et al., 2012).

The first data on seroprevalence of canine babesiosis in Hungary in 2006 announced Hornok, Edelhofer and Farkas (2006). They determined the seroprevalence of 5.7% using the indirect immunofluorescent antibody test. This is notably lower value than obtained in this study, as well as results reported in Albania (9.9–13.0%) and Sicily (5.17%) (Hamel et al., 2009; Lazri et al., 2008; Torina and Caracappa, 2006). In Slovakia reported seroprevalence ranges from 4.8% in Nové Zámky to 28.8% in Komárno (Kubelová et al., 2013) that corresponds to our results (from 13.7 to 35.0%).

Similar results to ours were reported by Imre et al. (2013): in dogs in Romania where seroprevalence value was 19.8%. Furthermore, they found that the seroprevalence of infection caused by *B. canis* was significantly higher in hunting dogs and dogs from rural areas compared to the population of dogs that live in different conditions, in kennels and the owners houses respectively. Looking at the values of the seroprevalence of this infection in different populations of dogs, it can be concluded that they are similar in hunting dogs and dogs that live on the street, which corresponds to observations by the authors from Romania, considering that a large part of the sera in a group of stray dogs from our research originate from rural areas. That dogs in shelters and street dogs, who are in close contact with ticks are more likely to become infected with babesias compared to dogs kept as companion animals indicates Cassini et al. (2009). This observation corresponds to our findings; lowest seroprevalence of canine babesiosis (13.7%) was in the population of companion dogs, which mainly live in the house and have a known owner due to the timely removal of ticks from the body of dogs and the application of repellents.

Greater exposure to the vectors of the hunting and stray dogs is a possible reason for the higher values of seroprevalence of canine babesiosis infections obtained in our research in comparison with the companion dogs.

Furthermore, divergence in the results obtained in our research and in previous studies conducted in Serbia indicates the necessity of more extensive seroepidemiological and molecular studies of canine babesiosis.

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STUDY OF THE GENETIC VARIABILITY OF THE PORCINE CIRCOVIRUS TYPE 2 IN UKRAINE

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Summary. Porcine circoviral infection is the economically significant and contagious porcine disease, affecting of the animals from different ages and causing multiple syndromes associated with immunodeficiency. The aim of this work consisted in the study of genetic polymorphism of the *Porcine circovirus* type 2 strains circulating in different farms in Ukraine to determine their genotype.

Study was performed by the molecular (PCR, sequencing) and bioinformatical (multiple alignment, phylogenetic study) methods. Samples were collected using basic methods from the productive pig farms in Eastern Ukraine. *Rep* gene 421 bp region has been used as the matrix for sequencing and phylogenetic analysis.

The 6 characterized isolates were related to 1 and 2 genotypes of PCV-2. They demonstrated different levels of variability in the comparison with strains from European, Asian and American origin, allocated in different countries.

The study of the PCV-2 genetic differences in 421 bp rep gene loci demonstrated relation of isolates to American-origin contaminants of the veterinary preparations — genotype 2, and other field strains were related to European progenitors of genotype 1 (70–93% homology).

Keywords: *Porcine circovirus* type 2, genotype, PCR, phylogenetic analysis.

Introduction. Porcine circoviral infection is a mainly contagious and highly distributed disease of domestic pigs and wild boars. It could be characterized with the development of the system immune deficits, disorders in the digestion and reproductive system, respiratory tract. They could be potentially complicated by the secondary pathogens.

The disease causative agent is *Procine circovirus* type 2, causing Post-wearing multisystem wasting syndrome (PMWS). Circoviral infection is the main disease in commercial and back-yard farms in every country with developed pig-breeding since 2001–2002.

Virus could potentially infect porcine fetus, because it easily migrates via trasplacentary barrier. The colostrum immunity, formatted just after born of the pigs, provide the protection during first 1–2 months of life.

However, after the transferring of the piglets to 2–4 group, when the maternal antibodies level decreases, their own immune system is blocked by circovirus that allows the agents of other viral infections (PRRSS virus, *Porcine parvovirus* etc.), bacterial infections including mycoplasmoses to develop the multiethiological disorders (Anon., 2005; Kovalenko et al., 2005; Orlyankin, Aliper, and Nepoklonov, 2002).

Circoviruses also could affect the adult pigs. It is characterized by development of the reproductive disorders (aborts, dead-birth, birth of the weak piglets etc.). In addition, the literature contains descriptions of infecting the adult animals with the development of the systemic immunodeficiency, which in several cases could be the reason of animal death (Anon., 2005).

Postweaning multisystem syndrome is described in European countries, United States of America, Canada, Brazil, former USSR member states and others. Annual economic losses, caused by PMWS, represent amount of 300 $ millions only in USA (Choi, Chae and Clark, 2000).

Porcine circoviruses are presented by two types — PCV-1 and PCV-2. PCV-1 is apathogenic. PCV-2 is the pathogenic agent, which is more interesting from veterinary medicine point of view. This virus causes the multiple lesions in pigs and piglets, and requires the effective means for diagnostics and surveillance (Choi, Chae and Clark, 2000).

The wide spectrum of the laboratory diagnostics techniques are used for virus detection and characterization in the veterinary practice. The list includes classical virology with isolation of virus in susceptible cells (PK-15), and further identification of the agent using ELISA and in-situ hybridization techniques (Kritas et al., 2007; Allan et al., 2007; Shkayeva et al., 2006; Blotska, 2008).

The different types of ELISA and PCR techniques are used for PCV-infection monitoring. PCR has been recognized as better technique for PMWS surveillance. It is used in classical and real-time modification for detection of virus-keeper animals (Fort et al., 2007; Shang et al., 2008).

Also the multiplex PCR-based protocols has been described to detect circoviruses in complex with other porcine pathogens, including Porcine parvovirus, Aujeszky's disease virus, African swine fever virus and others (Anon., 2007; Kim and Chae, 2001; Lee et al., 2007; Giammarioli et al., 2007).

The method of sequencing of the amplified viral DNA fragments is the one of the most informative techniques for molecular diagnostics and epithology of PMWS. The first genotyping studies were performed by Stevenson et al. (2001) and Allan et al. (1999). These researchers demonstrated presence of two genotypes of PCV-2 based on partial sequences of its genome. The divergence of sequences has been observed in the level of 4–5%. Also they described the correlation between genotype and clinical and pathological signs of the disease.

Two genetic lines (1 and 2) were described after full-length genomic sequencing of the viral DNA. The a and b sublines were detected in each line. Genotype 1 is presented mostly by European PCV-2 isolated, and genotype 2 includes American and Asian viruses. The genetic divergence among genotypes is about 3–5%. The differences are also 0.8–1.3% in genomes of 1a and 1b clades of strains (De Boisseson et al., 2004).

Molecular genetic tests for indication of viral DNA of circoviruses could be characterized as the most effective surveillance and diagnostics. Almost the wide disease spread in Central European

countries determines the necessarily of study of the virus genetic diversity for its characterization from genetic point of view.

The aim of this study was determination of the phylogenetic relations of Ukrainian PCV-2 isolates.

Materials and methods. Amplification of the variable fragment of circoviral DNA in the 421 bp region of *rep* gene was done using PCV-2_F/R primer set, early developed in NSC 'IECVM' [PCV-2 PCR protocol, 2011].

Sequencing was managed by SeqLab GmbH (Göttingen, Germany) and Lohmann Animal Health (Cuxhaven, Germany) by the classical Sanger method.

Sequencing chromatograms were edited using nucleotide sequences manager BioEdit v. 7.1.1.5.

Phylogenetic analysis of the rep gene variable loci was managed by MEGA v. 4.0. software, using neighbor-joining algorithm. Graphical analysis of the constructed trees has been done by TreeView v. 1.6.0.

PCV-2 *rep* gene sequences were published in GenBank sequences database (access numbers: EU275761, EU260051-54, EU252693).

Results of study. In order to establish the molecular characteristics of epizootic PCV-2 strains, detected in farms Ukraine, and study of their phylogenetic relationships with viruses isolated in different regions of the world our studies of DNA the sequencing of PCV-2 in 421 bp area of *rep* gene has been done. This gene is the most representative in terms of molecular epizootiology of porcine circoviral infection. DNA amplification products of PCV-2 *rep* gene 421 bp region were received using PCR. Amplicones has been derived from the material from pig farms in Kharkov, Lugansk, Poltava, Dnipropetrovsk and Belgorod regions. They were purified in agarose gels of impurities nonspecific DNA length. Further, these fragments excised and extracted from the gel method destruction zircon sand and elution of DNA in TE buffer. In order to concentrate DNA elution volume of 50 ± 4 ml dried at 65 °C.

Prepared fragments were assigned names: PCV2 Poltava (amplicon of viral material AF 'Fisun'), PCV2 Lugansk (LLC 'Granum'), PCV2 Lugansk2 (LLC 'Call'), PCV2 Kharkov (AF 'Dubrava'), PCV2 Dnipro ('YUSOLS') and PorBelgorog (FDE Krasnoyaruzkyy SC).

In the first phase were calculated DNA concentration after dilution, samples standardized within 44.2–54.4 mg/cm^3. Sequencing amplification primers carried out in the application system development PCV_1–2 NSC 'IECVM' and standard protocol.

The obtained sequences were analyzed by multiple alignments technique. As a result, the presence of three isolates clades in Ukrainian isolates of porcine circovirus type 2.

First clade, which included isolates PCV-2 Poltava and PCV-2 Dnipro compared to isolate k43 °C (for comparison matrix proposed Gagnon CA) had three mismatches: C → G at locus 308 bp, A → G — → T and 363 G — 365 bp. Second group of viruses included isolates PorBelgorog and PCV-2 Lugansk. These isolates had 11 substitutions in the study area rep gene and deletion of 2 bp size. The third clade isolates: PCV-2 Lugansk2 and PCV-2 Kharkov. They carried 14 nucleotide substitutions in the area rep gene 421 bp in length, but not adequately deletion at positions 198–199 bp by matrix alignment. Character of the replacements in second and third groups diverse loci has been presented 248 and 256 bp (not in group 2), 155, 289, 291, 367, 382 bp (not in group 3) (Fig. 1).

After broadcasting the resulting alignment second clade of viruses had 13 amino acids differences in comparison with the first group (isoleucine (I) → serine (S) at position 52, phenylalanine (F) → S — 53, 55, XX (reading frame shift due to deletions) — valine (V), asparagine (N) — 67, 66, arginine (R) → lysine (K) — 85, R → proline (P) — 103, glycine (Q) → leucine (L) — 107, tryptophan (T) → I 113, V → L — 121, 122, S → N — 123, R → K 130) with almost 140, coded sequence sequenced (Fig. 2).

Group 2 differs from 1st group only in five amino acid positions, and thus only five nucleotide substitutions identified in sequencing were significant (S → F — 55, R → K — 83, L → methionine (M) — 86, V → L — 94, N → S — 123).

Thus, it was established three subgroups virus circulation with a different nucleotide structure relative to each other. This in turn reflected and amino acid sequences of the gene *rep* studied viruses.

After analyzing the genetic variability of the pathogenic PCV-2 DNA, concluded that isolates the second group stimulated the development of respiratory disorders, while two other groups of viruses found in both reproductive and respiratory and when mixed forms of circoviral infection in pigs and piglets.

Analyzing the previous literature described and our own data, it is possible to make the solution that correlation does not always exist between *rep* gene sequence and pathogenic potency of virus (PCV-2). However, analysis of this gene provides us with enough data for study of the phylogenetic connection among the PCVs strains. That was the reason for upcoming phylogenetic study on the next stage of our research.

The Neighbor Joining algorithm in MEGA software has been used for the genetic analysis of PCV-2 strains circulation in pig farms in Ukraine. The rooted phylograms were constructed, and the *rep* gene sequence of canary circovirus has been used as the polarization sequence (Fig. 3).

Isolates PCV2 Poltava and PCV2 Dnipro were situated on the same cluster as early described isolate k43 °C of PCV-2, which is the potential contaminant of the cell lines. This circumstance describes the possibility of cultural origin of the mentioned viruses. It could be possible, that named populations of virus have infected animals via contaminated vaccines and immune-therapeutic preparations.

Other group of viruses on the dendrogram was presented by two subgroups, respectively, presenting the 2nd and 3rd cluster of the analyzed viruses.

The next step of our research was devoted to comparison of the sequences, derived from PCV's DNA samples, belonging to different isolates. This has been done using Kimura's comparison. The calculated data demonstrated the absence of the divergence in the 1st group of viruses, inside the 2nd group is was 1.7%, and for 3rd group – 0.2%. The distances between 1st and 2nd groups were 0.032–0.037, and the distances between 2nd and 3rd — 0.017–0.034, and 0.037–0.037 — between groups 1 and 3 respectively (Fig. 4).

The analysis of the comparison of neutrality evolution indexes by the Nei-Gojobori method demonstrated the high level means of the p-distances among viral groups (over 1.2). The neutrality test by Tajima method allowed to describe 19 segregation sites with segregation indexes (pS) 0.045346, conservatively index (pi) was 0.022730. Pairwise and evolutional indexes demonstrated the likelihood of the analyzed viruses in the frames of the *rep* gene analysis (Fig. 5).

Figure 1. The multiple alignment of PCV-2 sequences derived from 421 bp *rep* gene fragment from Ukrainian isolates of the agent

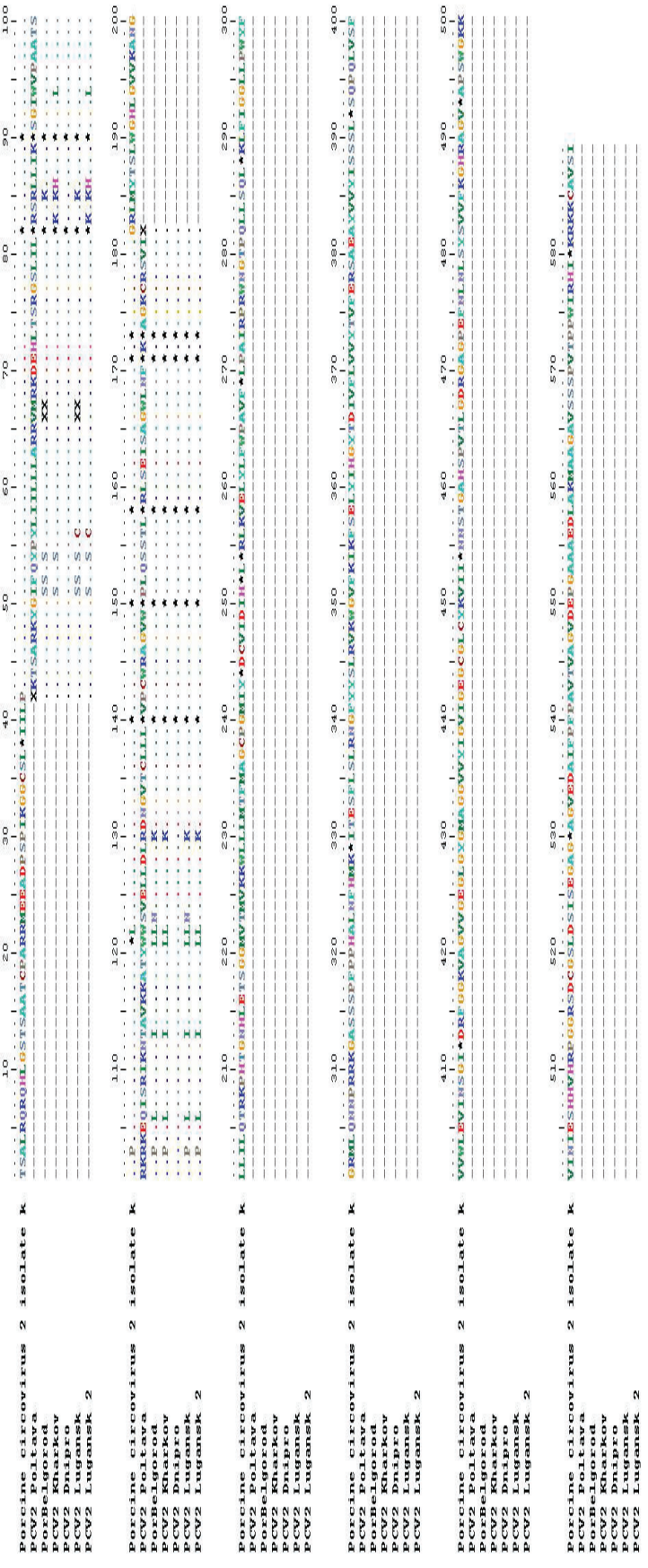

Figure 2. Amino acid sequence, derived from the sequenced 421 bp region of the replicative protein of the Ukrainian PCV-2 isolates

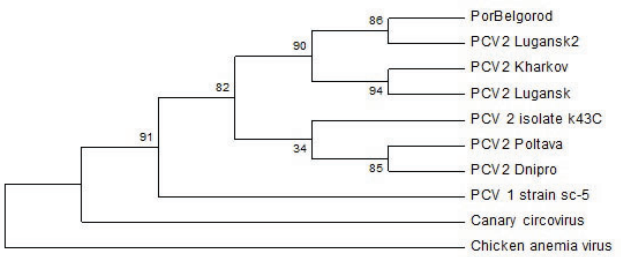

Figure 3. Rooted dendrogram of the Ukrainian PCV-2 isolates (*rep* gene)

The last step of our study included the creation of the phylogenetic trees from PCV-2 sequences of *rep* gene, allocated in the different locations using Neighbor Joining and Minimal evolution methods. The dendrograms were constructed using sequences, published already in the GeneBank. The out-group (canary circovirus and infectious chicken anemia virus, and PCV-1) sequences were used to create the rooted tree.

Pairwise Distances (G:\Porcine circovirus\PCV2 UA all seq.meg)

File Display Average Help

(A,B)

	1	2	3	4	5	6	7
1. PCV 2 Dnipro							
2. PorBelgorod	0.032						
3. PCV 2 Kharkov	0.034	0.017					
4. PCV 2 Poltava	0.000	0.032	0.034				
5. PCV 2 Lugansk2	0.037	0.019	0.002	0.037			
6. PCV 2 Lugansk	0.034	0.002	0.019	0.034	0.017		
7. PCV 2 isolate k43C	0.010	0.027	0.029	0.010	0.032	0.029	

(PCV 2 Dnipro-PCV 2 Dnipro) / Nucleotide: Kimura 2-parameter

Figure 4. The distances between PCV-2 Ukrainian isolates (*rep* gene)

The created tree (Fig. 6) demonstrated belonging of the 1st group of analyzed Ukrainian viruses to the lineage of the American strains contaminant of the cell lines of genotype 2 PCV-2 (88%).

The group contained the isolates PorBelgorod, PCV2 Lugansk 2, PCV2 Kharkov and PCV 2 Lugansk belonged to genotype 1 strains with European origin (similarity 70–93%).

Test of Neutral Evolution (G:\Porcine circovirus\PCV2 UA all ...

File Display Help

(A,B)

	1	2	3	4	5	6	7
1. PCV 2 Dnipro		0.309	1.234	0.000	1.078	0.479	0.336
2. PorBelgorod	0.758		1.256	0.309	1.044	1.081	0.622
3. PCV 2 Kharkov	0.220	0.212		1.234	1.080	1.044	1.876
4. PCV 2 Poltava	0.000	0.758	0.220		1.078	0.479	0.336
5. PCV 2 Lugansk2	0.283	0.299	0.282	0.283		1.261	1.721
6. PCV 2 Lugansk	0.633	0.282	0.299	0.633	0.210		0.444
7. PCV 2 isolate k43C	0.738	0.535	0.063	0.738	0.088	0.658	

(PCV 2 Dnipro-PCV 2 Dnipro) / Codon: Nei-Gojobori method (p-distance)

Figure 5. Neutrality evolution indexes in PCV-2 rep gene sequences comparison

The Minimal evolution method derived tree (Bootstrap 500) demonstrated lower likelihood concerning belonging of the 1st group isolates (only 25 and 54 % after condensation) (Fig. 7).

This could be explained by the big amount of genetically similar isolates that have different mismatches in the nucleotide sequences of the rep gene.

Isolates PorBelgorod and PCV2 Lugansk were situated also in the clade of the European viruses, and the common progenitor of them has been detected (am8 isolate). The Lugansk 2 isolate (82%) cold be potentially changed virus with the same origin and PCV2 Kharkov isolate.

The strains from the American genotype were mostly genetically related to am2 and am9 strains.

Conclusion. The study of the phylogenetic relations of PCV-2 Ukrainian populations based on the analysis of 421 bp region sequencing of the rep gene demonstrated their belonging to the lineage of the American origin cell cultures contaminated strains (88%) of the genotype 2. The group of sequences, contained DNA-amplicones of PCV-2 isolates PorBelgorod, PCV2 Lugansk 2, PCV2 Kharkov and PCV 2 Lugansk belonged to the lineage of European origin strains of genotype 1 (70–93%).

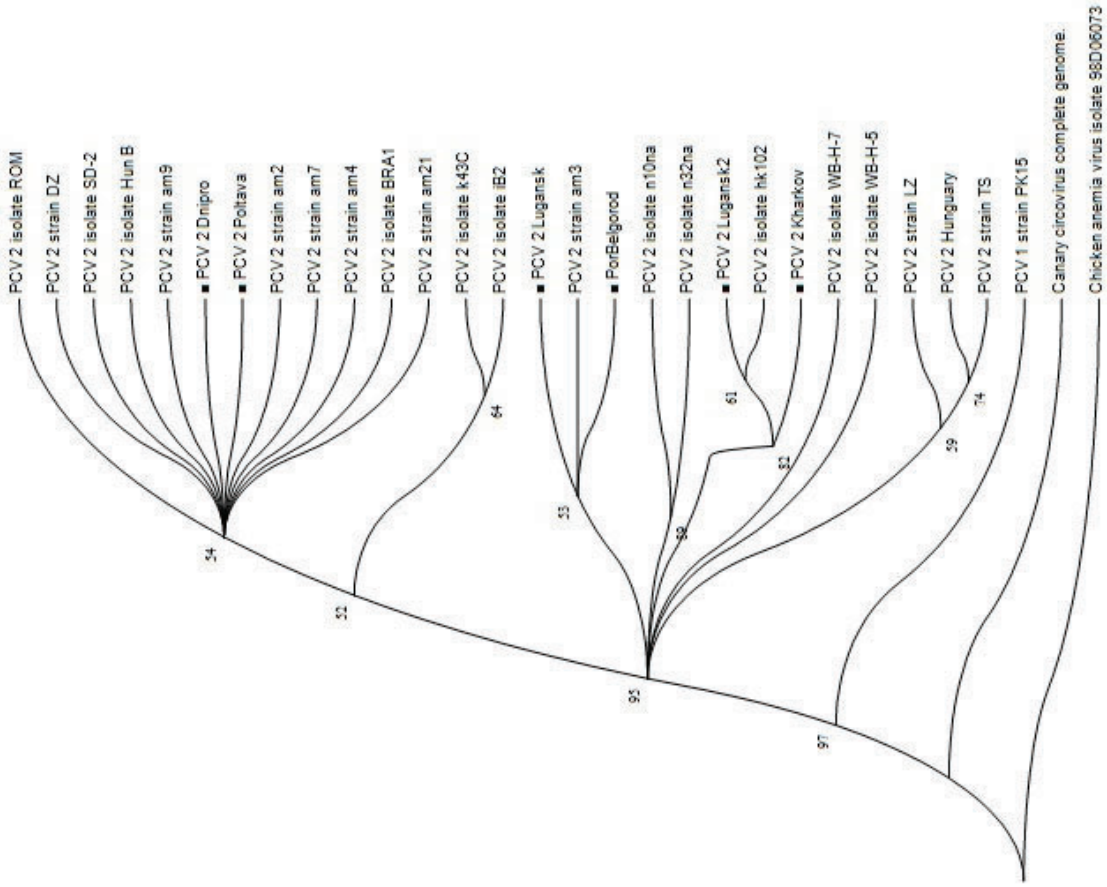

Figure 7. Condensed dendrogram of the phylogenetic relations of PCV-2 isolates and strains (Minimal evolution, ■ — Ukrainian isolates).

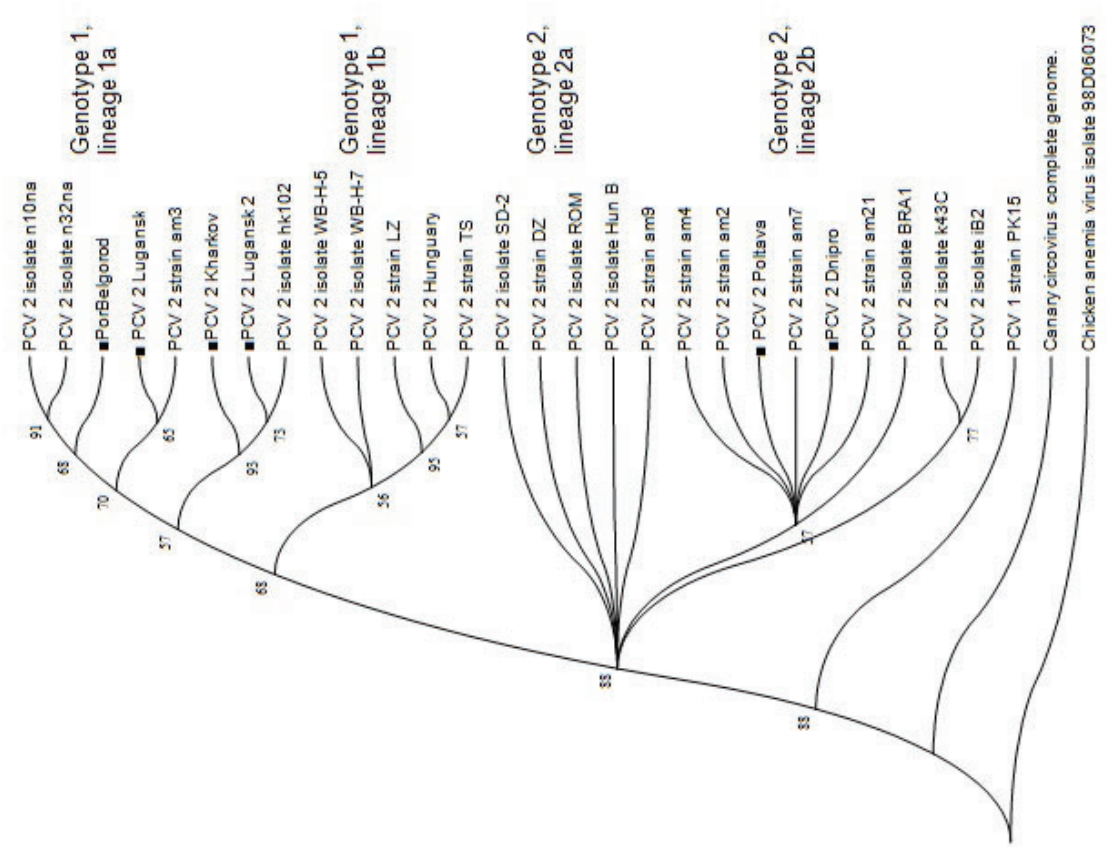

Figure 6. Dendrogram of the phylogenetic relations among the strains and isolates of PCV-2 (Neighbor Joining, ■ — Ukrainian isolates)

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DEVELOPMENT OF SOFTWARE FOR CALCULATING THE BIOLOGICAL ACTIVITY OF MYCOBACTERIAL ALLERGENS

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Summary. The paper contains the results of the development and production testing of the software tool calculation for biological activity of mycobacterial allergens for veterinary use. Purpose — to develop the software for calculating of the biological activity of mycobacterial allergens, and conduct it's testing of industrial development in the catch during the control of commercial series of tuberculin purified (PPD) for mammals (n = 28).

Software design system was used on *cba* platform .NET Framework 4.5, the programming language C# 5.0, the development environment Microsoft Visual Studio 2013, as well as WPF technology. Clinical studies were performed under conditions of Sumy State Biofactory by the parallel calculation of tuberculin purified activity (PPD) for mammals in the standard solution (series number = 28) using the developed program cba and calculation method of biological activity according to the current TC U 24.4-00497087-107:2011 'Tuberculin dry purified (PPD) for mammals (National standard)'.

As a result, the calculation of the biological activity of 28 experimental series of tuberculin for mammals according to the procedure specified in regulatory documents and with the help of the developed software *cba* demonstrated activity of the preparation at the level 53 746 U/cm^3 in standard solution.

The computer program *cba* for the determination of the biological activity of mycobacterial allergens provides accurate and timely calculation of the amount of unit content in the volume 1.0 cm^3 of the standard solution of purified tuberculin (PPD) for mammals.

Perspectives for future research will include conduction of the further testing of the software for allergen dry purified from atypical mycobacteria (AAM) and avian purified tuberculin (PPD) in the production environment, as well as in the development of targeted programs for different operating processes that require complex mathematical calculations in the manufacture process of different preparation dilutions, as the conditions of the producing and for research purpose.

Keywords: biological activity, software, units (U), mycobacterial allergens, tuberculin.

Introduction. Tuberculosis remains to be a dangerous anthroponotic disease requiring special attention in order to prevent more widespread. The main method of lifetime diagnostics of animals tuberculosis today is the skin allergic test performed using mycobacterial allergens (PPD tuberculin for mammals, avian PPD tuberculin, allergens derived from the atypical mycobacteriums (AAM)) (Kozlov, Bezgin and Shumilov, 2004; Kozlov, 2007).

One of the most important parameters of mycobacterial allergen quality, along with a specificity, sterility, environmental safety is their biological activity, which is measured in international units — 'U'. Provided excessive activity of allergen are manifestations of allergic reactions in healthy animals, and vice versa, if the biological activity of the drug is low — with TB animals do not react to tuberculin, which leads to the further spread of tuberculosis infection and, as a consequence, an increase in terms of improvement of disadvantaged households (Zavgorodniy et al., 2006). Therefore, it is very important to the accuracy of the calculation of this indicator in the production of commercial series mycobacterial allergens. Development of the target software for calculating the amount of U/cm^3 of the finished product allows standardization and speed up of the quality process control and support the elimination of possibility false estimation caused with 'human factor'.

The study aimed to develop the software for calculating the biological activity of mycobacterial allergens and conduct testing of developments in production conditions during the commission of control of commercial series tuberculin purified (PPD) for mammals (n = 28) on Sumy State Biofactory.

Materials and methods. The system *cba* was used developing software .NET Framework 4.5 platform, the C# 5.0 programming language, the Microsoft Visual Studio 2013 IDE, as well as WPF technology were used (Troelsen, 2013; Richter, 2013). Clinical studies were performed under conditions of Sumy State Biofactory by the parallel calculation of tuberculin purified activity (PPD) for mammals in the standard solution (series number 28) using the developed software *cba* and calculation method of biological activity according to the current TC U 24.4-00497087-107 'Tuberculin dry purifications (PPD) for mammals (National standard)' (2011).

The results of the work. As a result, the calculation of the biological activity of the experimental series of tuberculin for mammals (n = 28) according to the procedure specified in regulatory documents (TC U 24.4-00497087-107, 2011), and with the help of the developed software *cba* (Fig. 1, 2) demonstrated that the activity of the preparation was on the level of under using of the 53 746 U/cm^3 of standard solution.

Figures 1 and 2 demonstrate the performance of the calculation of the biological activity of examined preparation — tuberculin for mammals (28). In order to determine the activity of the test preparation in international units (U) must be made in cells # 1, 2, 4, 5, 7, 8, 10 and 11 (Fig. 1) the magnitude of the dimensions of papules guinea pigs (mm) of each individual formed in 24 hours after administration of the preparation in 4 dilutions — first dilution: 1 and cell 2 II (4, 5), III (7, 8) and IV (10, 11). The cells # 3, 6, 9 and

12 respectively defined by the average value of allergic reactions in laboratory animals in each of 4 dilution of the preparation. The system then calculates the index cba bioactivity, which is reflected in the corresponding panel (Fig. 2). The principle of software calculation corresponds to the method of calculation according to TC U 24.4-00497087-107:2011. In addition, the program has the ability to comparison of this indicator up to 3 series products at the same time reflecting the results in graphical form, as shown in Fig. 1. Thus, the developed software cba using allows to found that the activity of a series of tuberculin for mammals (n = 28) of Sumy State Biofactory is 53,746 U/cm^3 of standard solution. This figure is identical to the measure of biological activity specified in the certificate of quality on this series of tuberculin for mammals.

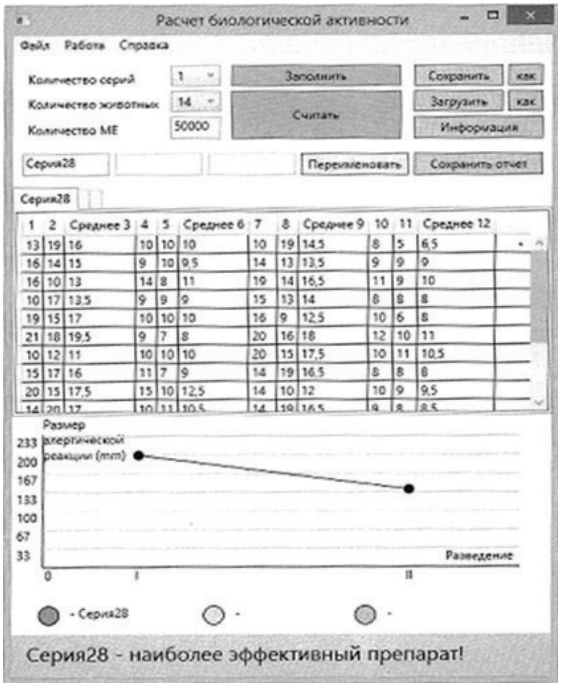

Figure 1. The calculation panel of the biological activity of tuberculin for mammals (screenshot in Russian)

Conclusion. The cba software for determination of the biological activity of mycobacterial allergens provides accurate and timely calculation of the U/cm^3 of standard solution of purified tuberculin (PPD) for mammals.

Prospects for further research are to conduct further testing of this software for allergen dry purified from atypical mycobacteria (AAM) and avian tuberculin purified (PPD) in the standard solution in a production environment, as well as in the development of software for different operating processes that require complex mathematical calculations in the manufacture of various products, the preparation of the dilutions in the conditions of production, and for research purpose.

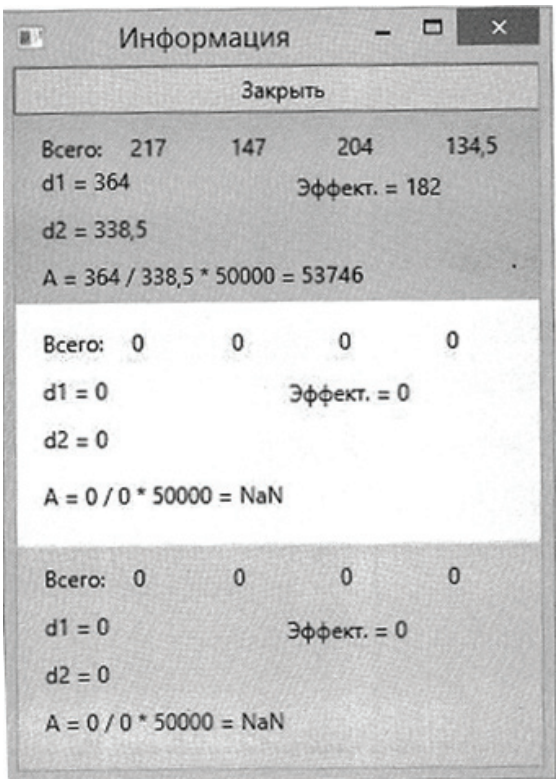

Figure 2. The calculation panel results of the biological activity of tuberculin for mammals (screenshot in Russian)

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Part 2. Veterinary medicine