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THE DYNAMICS OF THE SPECIFIC IMMUNITY FORMATION IN SHEEP UNDER THE INACTIVATED VACCINES AGAINST CONTAGIOUS AGALACTIA OF SHEEP AND GOATS

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Summary. The aim of the research is constructing and efficiency studying of inactivated vaccine against contagious agalactia of sheep and goats.

The bacterial mass of *Mycoplasma agalactiae* S-11 production culture, obtained on artificial media, has been used as initial product. The vaccine contains 60% of formalin-inactivated mycoplasmas cell suspension (6×10^7 cfu in a dose) in sterile phosphate-buffered saline and 40% of aluminum hydroxide. Vaccination of sheep in the sheep farms of Odessa region by 'Ahavak' vaccines (S.N. 'Institutul Pasteur' S.A. Romania) and NSC 'IECVM' using different schemes. Animals were studied using bacteriological, serological and biochemical methods.

The vaccine from *Mycoplasma agalactiae* S-11 strain does not cause the local reaction and immunosuppression on organisms of sensitive livestock animals. The level of protective antibodies among research groups of animals for NSC 'IECVM' vaccine was 3.54 (3.47–3.61) $\log_2$ at the 30th day after revaccination and 3.59 (3.52–3.64) $\log_2$ for 'Ahavak' vaccine. The antibodies against *Mycoplasma agalactiae* were registered in 14 heads of the control group (n=50) and accounted for 1.31 (0.00–2.86) $\log_2$.

It was found that disease signs and presence of pathogen are absent in conjoined holding animals which were vaccinated against contagious agalactia of sheep and goats by NSC 'IECVM' and Romanian vaccines as compared to the animals with clinical sings of the disease. The intracutaneous injection of inactivated vaccine against contagious agalactia of sheep and goats (NSC 'IECVM') two times provides 100% protection of sensitive animals against clinical signs of the disease. It is harmless, areactogenic, with immunogenic and protective properties equally to the Romanian vaccine.

Keywords: contagious agalactia, sheep, goats, inactivated vaccines, ELISA

Introduction. Contagious agalactia of sheep is widespread in countries with developed sheep farming (Turkey, Spain, Italy, etc.). The pathogen (*Mycoplasma agalactiae*) can circulate in group of sensitive animals during some years. The disease runs in subclinical form, but if there is stock infection more than 70%, the outbreak of clinical signs of disease happens (the peak season falls at lambing). The most sensitive are animals under lactation and young ones. Nowadays, the contagious agalactia is registered in some districts of Odessa region in Ukraine. However, due to the fact that sheep farming is actively developing, the disease can spread in other regions also. The developing of homeland biological products for diagnosis and preventing the contagious agalactia is an actual field of research (Ariza-Miguel, Rodríguez-Lázaro and Hernández, 2012; Kumar et al., 2014; Madanat, Zendulková and Pospíšil, 2001; Poumarat et al., 2016; *Veterinary Record*, 2014).

According to the OIE, the commercial vaccines against contagious agalactia of sheep, caused by *M. agalactiae*, and inactivated with formalin, are widely used in South Europe. Some researches consider that

they are ineffective. In vitro, the vaccines against *M. agalactiae*, inactivated with saponin or phenol, have more protective effect that formalized ones. Alive vaccines against *M. agalactiae* are used in Turkey, where they are more efficient, as it is reported, than inactive ones.

Materials and methods. The bacterial mass of *Mycoplasma agalactiae* S-11 production culture, accumulated in liquid culture media during 3 serial passages (72 h at 37°C), was used as an initial product. Vaccine was obtained by the following method: bacterial mass of the micoplasmas was inactivated by adding 1% formalin (24 h at 37°C). Inactivated cells were precipitated by centrifugation and washed twice with sterile PBS (centrifugation mode 3 ths.rev./min for 20 min). The suspension with 1×10^8 cfu/sm^3 concentration was prepared. Aluminum hydroxide is added to bacterial mass. The component ratio of vaccine is following: 60% of *Mycoplasma agalactiae* production strain inactivated cell suspension (6×10^7 cfu in one dosage) in sterile phosphate-buffered saline and 40% of aluminum hydroxide.

Experiments on sheep conducted under production conditions in sheep farm of Odessa region. There was dedicated a group of 150 animals from a herd. It was reformed in three groups with 50 animals in each one:

1. Intact group (50 animals) with no vaccination.

2. Research group (50 animals) vaccinated with inactivated vaccine against infectious sheep and goal agalactia (NSC 'IECVM') intracutaneously in the tail fold twice in the dosage of 1 sm^3 per 30 days.

3. Research group (50 animals) vaccinated with inactivated vaccine against infectious sheep and goal agalactia 'Ahavak' (S.N. 'Institutul Pasteur' S.A., Romania) intracutaneously in the tail fold twice in the dosage of 1 sm^3 per 30 days.

The blood of all animals was sampled for serological and biochemical studies before the immunization and on 30th days after second vaccination. The biological material (swabs from nares and eyes, milk samples) of animals from both groups was sampled and studied with bacteriological method for *Mycoplasma* presence before experiment beginning. The γ-globulins level in blood serum samples of intact and research groups was detected using standard methods. Nasal and eyes swabs were sampled from 10 animals in each of three groups for bacteriological studying on the presence of infectious agalactia pathogen at the end of the research.

Results. The clinical signs of disease and side-effects in the place of injection (reddening, swell) were absent during the studying of *Mycoplasma agalactiae* S-11 formalin-inactivated epizootic culture protective properties on sheep. Mycoplasmas were extracted from two animals after bacteriological studying of biological material from investigated animals (nasal and eyes swabs).

We conducted biochemical analyses of sheep serum samples from intact and research groups for the purpose of studying the effect of vaccines at the general physiological animal condition. There were studied such factors as general level of proteins, seromucoids, circulating immune complexes and lysozyme. The research results are shown in Fig. 1–3.

The level of lysozyme, general amount of proteins and circulated immune complexes in serum of investigated animals increased when studying. We estimated potential immunosuppressive effect of vaccine on sheep by seromuciod status. The preparation had no immunosuppressive effect on sheep; the seromucoid level almost hadn't changed after vaccination in intact and research animals.

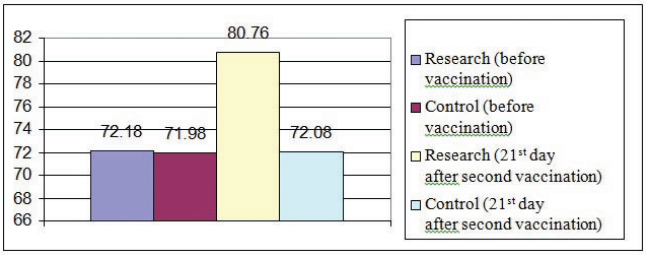


Figure 1. The dynamics of general protein level in sheep serum from control and research groups (g/l)

Injection of *Mycoplasma agalactiae* S-11 epizootic culture vaccine increased the level of general protein amount and circulating immune complexes in sheep serum of research group at 10% and 13% respectively.

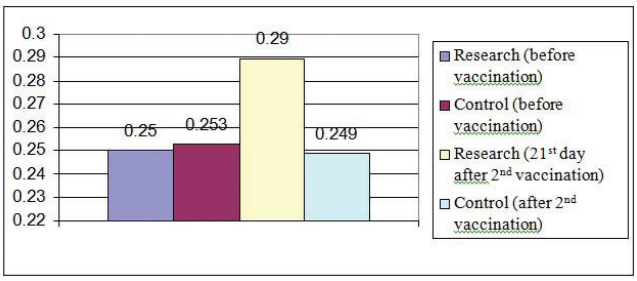


Figure 2. The changing level of circulated immune complexes in sheep serum of control and research groups (mg/ml)

The lysozyme level in sheep blood serum characterizes the level of non-specific antibacterial resistance of organism and it can have no direct connection with effects of immune medication. However, the results at the Fig. 3 show, that the lysozyme level among vaccinated animals increases. At that time the lysozyme level in control group has been lower,

that in the research one. We explain this fact that animal sensibilization with mycoplasma agent activates functioning of the specific and non-specific pathways intensity.

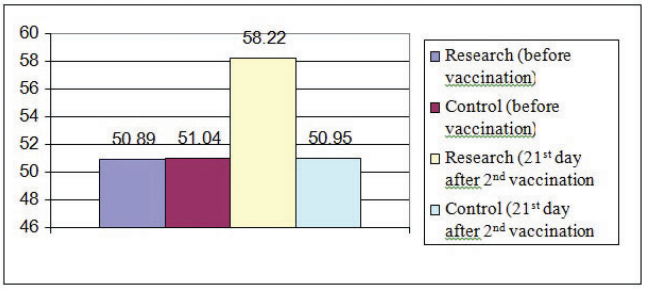


Figure 3. The dynamics of lysozyme level in sheep blood serum of research and control group (mkg/sm^3)

So, it has been determined, that experimental batch of vaccine from *Mycoplasma agalactiae* S-11 formaline inactivated culture is sterile and safe for anumals. It causes protection of 90% immunized animals after double vaccination in the challenge experiment with lethal dose of *Mycoplasma agalactiae* S-11 epizootic strain. Vaccine has no imunosupressive effect for animals and doesn't cause local allergic or inflammatory lesions in vaccinated animals.

To determine the immunogenic properties of the vaccine research was conducted on sheep in a production sheep-breeding farm in the Odessa region. Sheep were divided into groups and treated as planned experiment.

Biological material samples (nasal and conjunctive swabs, milk samples) from the experimental animals were examinated for infectious agalactia of sheep and goats agent by bacteriological method. The mycoplasmas were not detected.

After 30 days after the second vaccination of animals of all groups were selected for blood and serum biochemical studies. At the end of the experiment, 10 animals from each of the three groups were selected nasal and eye swabs for bacteriological research for the presence of infectious agalactia. In the group vaccinated animals for signs of disease were not found.

The reactogenic properties study of the vaccine against *Mycoplasma agalactiae* demonstrated their absence during all experiment period.

We occurred 12 sheep with clinical signs of suppression, watering, and swelling of the knee in the herd. Thick animals contacted with two groups of the vaccinated animals and intact sheep. At the end of experiment 5 sheep from the non-vaccinated animal group demonstrated general depression and watering. Both of them were treated by macrolide antibiotics, and the therapy demonstrated effectiveness.

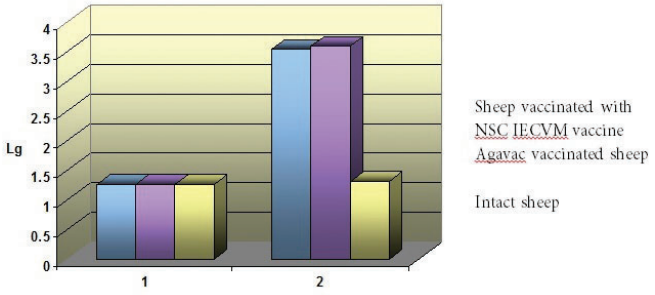
We didn't isolate agent from vaccinated animals after 5 blind passages of the clinical specimens from vaccinated animals. *Mycoplasma agalactiae* was isolated from 6 animals of the intact sheep group.

The study of immunogenic properties of *Mycoplasma agalactiae*, which is the main antigenic component of NSC 'IECVM' vaccine were tested in the experiment in sheep (Fig. 4).

The antibody titer among vaccinated animals was 3.54 (3.47–3.61) $\log_2$ for NSC 'IECVM' vaccine and 3.59 (3.52–3.64) $\log_2$ for vaccine 'Agavac' in 30 days after immunization. 14 intact animals of the control group (n=50) demonstrated antibody level 1.31 (0.00–2.86) $\log_2$.

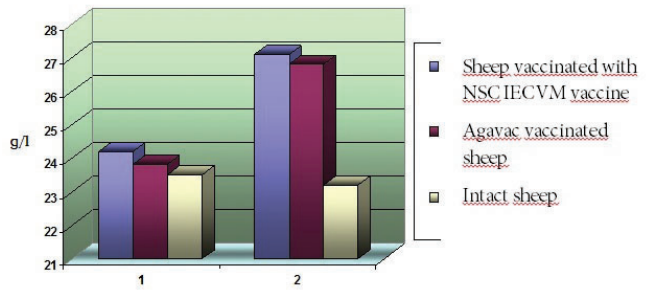
The vaccinated and intact animals' sera were also tested for biochemical parameters to study the non-specific immune response dynamics. We tested the level of γ-globulines before immunization and in 30 days after revaccination (Fig. 5).

It was demonstrated, that inoculation of both NSC 'IECVM' and 'Agavac' vaccined stimulated growing of γ-globulines levels for 10.7% and 11.1% respectively. At the intact animals group their level was more or less constant — 23.2 g/l.



1 – blood sera testing before experiment; 2 – blood sera testing at 30th day after immunization.

Figure 4. Antibody titers for infectious agalactia agent in blood sera of experimental animals of treated and control groups (Me, %25–%75, n=10)



1 – blood sera testing before experiment; 2 – blood sera testing at 30th day after immunization.

Figure 5. γ-globulines level in blood sera of vaccinated and non-vaccinated animals (Me, %25–%75, n=10)

The inactivated NSC 'IECVM' vaccine against infectious agalactia of sheep and goats is unreactogenic in conditions of the double subcutaneus administration. This preparation develop 100% protection in vaccinated animals from clinical disease.

Vaccinated animals by 'Agavac' (Romania) and NSC 'IECVM' vaccine, kept with non-vacicnated animals with clinical signs are protected from the infection.

Conclusions. The simultaneus keeping of animals vaccinated by 'Agavac' (Romania) and NSC 'IECVM' vaccine, and non-vacicnated animals with clinical signs demonstrates protection of immunized sheep from clinical infection with infectious agalactia. The double subcutaneus administration of the inactivated NSC 'IECVM' vaccine provide 100% protection of the susceptible animals. The preparation in safe, sterile, areactogenic and have immunogenic properties equal with Romanian vaccine 'Agavac'.

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COMPARATIVE ANALYSIS OF THE METHODS OF MOLECULAR DETECTION OF AVIAN INFLUENZA VIRUS SUBTYPE H5N1

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Summary. The article presents results of monitoring investigation avian influenza in birds of various kinds: synanthropic, wildlife, zoo, private sector and poultry farms during 2013. A comparative analysis of the molecular detection of avian influenza virus by polymerase chain reaction and isothermal nucleic acid amplification (RT-LAMP) conducted. Proved that RT-LAMP has a promising implementation in practice of veterinary medicine laboratories of Ukraine as express-method of diagnosis of avian influenza virus.

Keywords: avian influenza virus, diagnostics, epizootic situation, monitoring investigation, the method of isothermal nucleic acid amplification

Introduction. Avian influenza — a highly contagious viral disease that is characterized by high birds mortality (up to 100%). Etiologic agent that causes the disease — RNA-containing virus which belongs to the genus Influenzavirus, family Ortomyxoviridae. It is mainly spherical vibrios diameter of 80–120 nm polymorphic. The virus is a type A has 16 subtypes by subtypes of hemagglutinin and 9 — by neuraminidase. For most pathogenic avian subtypes are H5 and H7 (Pryskoka et al., 2014; Bakulov et al., 2002; Capua et al., 2003).

In Ukraine, the diagnosis of avian influenza carried out comprehensively considering epizootic data, clinical, and laboratory pathologists change research.

Influenza must be differentiated from other diseases of birds such as laryngotracheitis, Newcastle disease, respiratory disease (Golovko, 2007; Belousova, Trotsenko, Preobrazhenskaya, 2006).

Therefore, the method of diagnosis avian influenza virus put forward a number of requirements in indicators of specificity, sensitivity, reproducibility and duration analysis (Pryskoka et al., 2014; Golovko, 2007).

In laboratory diagnostics occupies a special place highly sensitive method of polymerase chain reaction. This method is based on the amplification of specific sections of the genome of a certain type of pathogen. High sensitivity, specificity and short time analysis make it promising in the diagnosis of avian influenza virus. Unfortunately, polymerase chain reaction analysis requires the use of expensive equipment and reagents and therefore not always available to laboratories that have resource constraints (Sidoti et al., 2009; OIE, 2012).

Important is the development of simple and sensitive rapid methods of diagnosis of avian influenza adapted to local conditions. One of these is a new approach that is based on isothermal nucleic acid amplification**.** In combination with reverse transcription LAMP suitable for amplification of RNA-matrix (RT-LAMP) (Ji et al., 2010; Shivakoti et al., 2010). We previously chosen reaction mixture and the reaction conditions optimized RT-LAMP for the diagnosis of avian influenza subtype H5N1 (Postoienko et al., 2013).

The aim of this work is testing in monitoring studies of our proposed method a RT-LAMP and comparative sensitivity analysis and the detection results of avian influenza by polymerase chain reaction in real time (PCR-RT) and RT-LAMP.

Materials and methods. In conducting a comparative analysis of results of detection of avian influenza virus subtype H5N1 by PCR-RT, RT-LAMP using experimental data obtained at the State Research Institute of Laboratory Diagnostics and Veterinary Expertise in the study of pathological material from birds, which arrived from all regions of Ukraine monitoring under the State Plan for 2013.

During PCR-RT were used commercial kits both domestic and foreign manufacturers, namely 'Bird-Influenza-PCR' (Ukrzoovetprompostach, Ukraine) and 'Quageen' (USA). Terms of amplification reactions and parameters given in the guidelines for use kits.

Terms RT-LAMP described earlier. In work applies the optimum temperature and reaction time — 59°C and 60 min (Postoienko et al., 2013).

The sensitivity of the diagnostic kit 'Bird Hrypp-PCR' and our proposed RT-LAMP determined by examining the cDNA reference strain of avian influenza H5N1, which provided National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine) in the concentration range from 10.0 to 0,01 ng per sample.

Results. For 2013 we studied 1,943 samples of material from birds by PCR-RT and RT-LAMP (Table 1).

Table 1 – Monitoring of avian influenza in Ukraine in 2013

Material type	The number of investigations by PCR-RT	The number of investigations by RT–LAMP
Pathological material from birds	692	692
Tracheal and cloacal wash	33	33
Sand	1	1
Birds's manure	1217	1217
Total	1943	1943

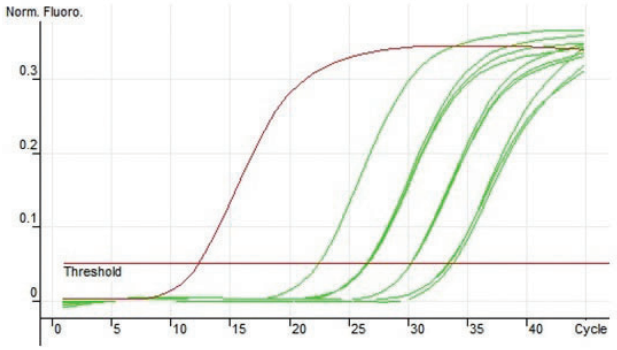
Monitoring of avian influenza among birds made of various kinds: synanthropic, wild, zoo, private sector and industrial poultry farms. The experimental data showed lack of avian influenza in studied samples. Also found that both methods use molecular detection of avian influenza virus, namely PCR-RT and RT-LAMP gives comparable results. But the practical use of RT-LAMP compared to PCR-RT has some significant advantages such as significant reduction of the duration of the analysis, the applicability of this approach in the field and lack of expensive equipment. Along with this, one of the important characteristics of a method is its sensitivity.

The indicator for PCR-RT was determined using a set of 'Bird-Influenza-PCR' (Table 2, Figure 1).

Table 2 – The sensitivity of PCR-RT test system 'Bird-Influenza-PCR'

QuantitycDNA, ng per sample	Type	Cross threshold	
		FAM	JOE
10	unknown	26.05	22.61
1	unknown	30.73	26.78
1	unknown	30.36	26.73
1	unknown	30.26	26.66
0.1	unknown	34.05	30.41
0.1	unknown	34.99	30.40
0.1	unknown	34.10	33.90
0.01	unknown	43.37	33.90
0.01	unknown	42.33	33.59
0.01	unknown	36.68	33.52
–	negative control	—	—
+	positive control	11.45	12.50

The number of cycles in the amplification channel FAM



The number of cycles in the amplification channel JOE

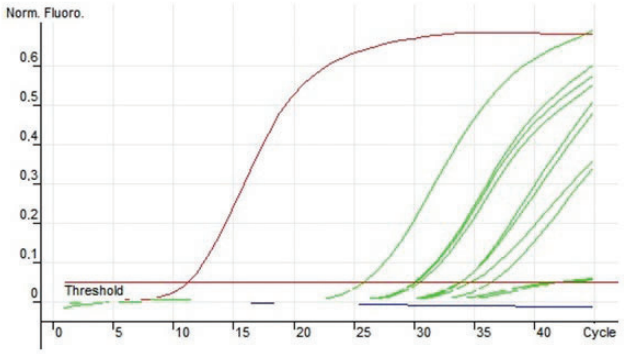


Figure 1. The sensitivity of PCR-RT test system 'Bird-Influenza-PCR'

The main criterion for evaluation of the results is to determine the threshold cycle (Ct), characterizing a stage PCR-RT, which observed a statistically significant increase in fluorescence compared to the background level. When using a diagnostic kit 'Bird Hrypp-PCR' samples were considered positive if the value of Ct through the channel FAM or where less or equal to 40 (St≤40), indicating the presence of gene amplification of influenza virus H5N1 found fragments respectively. Found that sensitivity of PCR-RT dial 'Bird-Influenza-PCR' is equal to 0.01 ng (FAM) and 0.1 ng (JOE) to test cDNA avian influenza virus type A subtype H5N1.

Sensitivity set developed by us during the research RT-LAMP method is 0.1 ng in the sample (Figure 2).

Slightly lower sensitivity of RT-LAMP is because in this method using visual detection reaction products. However, this figure corresponds to the world standards. The literature shows that the detection sensitivity of LAMP in avian influenza subtypes H5 and H7 equal to 0.1 ng per sample (Sidoti et al., 2009).

Found that sensitivity of both methods is high. Comparative analysis suggests a lower figure for LAMP, which may explain the ability of visual detection of the reaction products.

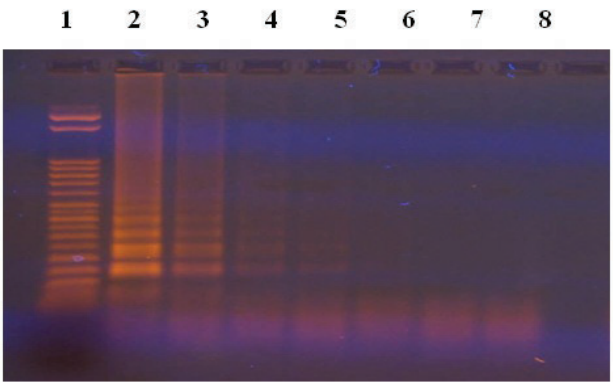


Figure 2. Electrophoretic detection products RT-LAMP, made with different concentrations of avian influenza virus DNA (ng prototypes): 1 — molecular weight marker, 2 — 10.0, 3 — 5.0, 4 — 1.0, 5 — 0.1, 6–8 — 0.01

Conclusions. 1. Comparative analysis found that using of both methods molecular detection of avian influenza virus namely PCR-RT and RT-LAMP gives comparable results confirming the promising method of rapid diagnosis of infectious animal diseases.

2. It is proved that the sensitivity of both methods of molecular detection of avian influenza virus meet international requirements.

3. Implementation RT-LAMP in the practice of veterinary medicine will enable timely control the entry of pathogens into the country.

4. Viability using RT-LAMP in the practice of veterinary medicine substantiated obtaining a number of significant advantages: short time of analysis, the applicability of this approach in the field without the use of expensive equipment.

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CLINICAL AND EPIZOOTIC CHARACTERISTICS OF DERMATOMYCOSIS MANIFESTATION AND THEIR ROLE IN NOSOLOGICAL STRUCTURE OF SKIN DISEASES IN DOGS AND CATS IN KHARKIV

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Summary: The article presents data concerning epizootic situation of dermatomycosis among small domestic animals (n=4280) for the observing period from 2005 to 2015 in two Kharkiv's veterinary clinics. The nosological profile of dermatitis in dogs and cats has been determined and it was presented by 7 factors with prevalence of dermatomycosis (61.4 %). Pet's disease incidence of dermatomycosis depending on age and breed susceptibility to illness have been analyzed. It was paid an attention to the importance of hematologic studies which point to the different skin pathology

Key words: dermatomycosis, dermatomycetes, diagnostics, dogs, cats, skin diseases

Introduction. For the last years skin diseases comprised about 30–70% of total small domestic animals pathology. Epiparasites, infectious agents, hypo- and avitaminosis, metabolism disorders cause the skin affection. One of the leading places belongs to microscopic fungi which can live on animals' skin for a long time or in association with other microorganisms can cause or increase inflammatory processes (Gryazin, 2001; Skrypnyk and Stetsiura, 2004). The biotic potential of various fungi pathogens, especially dermatomycetes, is high and their sources are widespread depending on the epizootic situation (Marchisio et al., 1995).

The results of clinical observations and experimental searches testify that all the aspects of immune system play an important role in pathogenesis of diseases. Discovery of T- and B-lymphocytes leading role in immune, their cell relation to macrophages, the elaboration of immune competent cells give the possibility to assess animal immune state objectively (Gordienko et al., 2007; Sigurgeirsson et al., 2002).

The aim of this work was to characterize nosological profile of dermatitis in dogs and cats.

Materials and methods. A retrospective survey of different skin disease cases registered among dogs (n=1733) and cats (n=1289) in two private veterinary clinics has been submitted to the Department of Epizootology and Veterinary Management (Kharkov State Zooveterinary Academy) from 2005 to 2015.

Clinical features of the disease were studied by clinical inspection of sick animals. The selection of materials for luminescent, microscopic and mycological investigation was performed from a lesion in the hairline, which had emerald — green radiance, or from a lesion in the hairline, and sometimes claws, with a characteristic formation of the skin hairless, sharply bounded circular spots covered with yellow-gray scales.

The biological material for microscopy and culture (on Sabouraud medium) research served as hair and skin scales from the affected areas of the skin of animals not subjected to treatment. The biological material for microscopic examination was taken from the periphery of the hearth by deep scraping scalpel. The test material was covered with cover glass, gently heated over the flame of a spirit lamp until the white rim from the crystals of alkali on the periphery of the drop and were subjected to microscopy using a light microscope.

Results. The analysis of small animals' incidence according to statistic data of clinics showed that 40.5% of the observed animals had skin pathology of various etiologies. Nosological structure of dermatitis in dogs and cats has been covered and presented by 7 factors among which dermatomycosis dominated. They have been found in 61.4% of animals with skin lesion (Fig. 1).

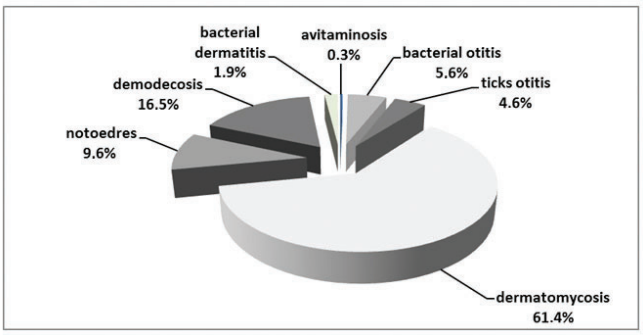

Figure 1. Etiological structure of dermatitis in carnivores

About 30.8—35.7% of dogs and cats out of the total amount of sick animals suffered annually from dermatomycosis in the observational period that has been defined during epizootic situation searching. There have been defined three animal groups according to the age peculiarities (Table 1).

Table 1 – Pet's incidence of dermatomycosis depending on the age

Age groups	Dogs			Cats		
	Total	Number of sick animals		Total	Number of sick animals	
		n	%		n	%
Young animals (< 6 months)	560	128	22.9	301	93	30.8
Adult animals (from 6 months to 7 years)	1459	437	29.9	1209	432	35.7
> 7 years	386	108	28.0	365	127	34.7
Total	2405	637	26.5	1875	652	34.8

The first group consisted of kittens and puppies from birth to 6 months age. Adult animals were united in the second group at the age from 6 months to 7 years. Animals of the third group were 7 years older. Studying breed susceptibility to dermatomycosis among cats authentic differences have not been found. Diseases have been recorded both in spread and rare cat breeds. Dermatomycosis has been found in 18 breeds of dogs out of 106 searched (Table 2). High incidence of disease has been recorded in French bulldogs, dachshunds and in nondescript dogs. Increased number of animals from May to August and decreased from September have been analyzed as annual dynamics of dermatomycosis occurrence in summer season. The maximum number of diseased dogs have been defined in August (20%) and cats in October (15%). Dermatomycosis manifestation in small animals is various. Acute and chronic cases of disease and also superficial, follicular, atypical, latent forms have been marked in observation period. Single and numerous hairless areas located on separate body parts such as muzzle, paws, back and sides have been noticed during dogs' examination. Single areas sometimes were mixed with the rest ones forming the complete surface of lesion.

Table 2 – Dogs' incidence of dermatomycosis depending on the breed

Breed	Total	Number of dermatomycosis cases	
		n	%
Bulldog	125	32	25.6
Airedale Terrier	124	25	20.1
Chow Chow	145	29	20.0
Dachshund	71	15	21.1
Spaniel	144	18	12.5
American Staffordshire Terrier	360	39	10.8
Dog	67	17	25.3
German shepherd	98	12	12.2
Pinscher	45	13	28.8
Rottweiler	61	17	27.8
Doberman	34	16	47.5
Boxer	78	16	20.5
Central Asian Shepherd Dog	147	16	10.8
Pekingese	59	15	25.4
Poodle	87	17	19.5
The Caucasian Shepherd Dog	29	9	31.1
Toy Terrier	40	4	10.0
Outbreed	139	16	11.5
Total	1853	326	17.6

Separate moist parts have been noticed in dogs with thick hair covering (Chow Chow, German Shepherds, Caucasian Shepherd Dogs, nondescript dogs, etc.) and with well-developed subcutaneous layer (Rottweiler

Dogs, American Bulldogs). In dogs with short coarse hair covering (Dachshund, American Staffordshire Terrier, French Bulldog, etc.) the hairless centers of infection with flux skin located on the back, in groin areas. Inflammatory reactions of various stages: skin irritation, itching, peels formation have been observed. During latent form of disease in dogs there have been diffusive hair dropping and sometimes dandruff.

Cats had non-symptomatic disease manifestation in form of long time fall-off. Hairless cats of rare breeds suffered from dermatomycosis in form of small separate centers of infection with irregular shapes with slight skin peeling on the distal areas and reddening in the central areas located on various body parts. Factors influencing dermatomycosis appearance in domestic animals have been found in the result of conducted investigation. Animal increase, crowded animal keeping, migration of infected animals from other regions, non-sanitary animal keeping and out-doors keeping influence dermatomycosis cases. Lack of movement, non-balanced ration in vitamins and mineral substances, inbreeding, groundless taking of antibacterial, vitamin and other preparations cause the decrease of skin protection functions.

In some cases the lack of efficient treatment has been noticed as lingering illness and complicated pathology. Dislocation of neutrophil nucleus to the left position, an increase of lymphocytes amount in 57.7%, basophils and eosinophils in 7.6% have been found during hematologic search of most examined dogs. These indices may tell us about susceptible pathology to skin diseases. Total urine analysis showed changes in mineral, protein and fat metabolism. Proteinuria has been noticed in 30% of dogs and 45% of cats, the presence of bile pigments (bilirubin, urobilinogen) in 86% of dogs and 85% of cats. The glucose presence in urine in 5% of dogs and in 2% of cats testified about pancreas disorders and the puiria presence in 63% of dogs and 31% of cats testified about excretory system disorders.

Conclusions. Using data from two Kharkov's veterinary clinics during 2005-2015 the incidences of skin diseases in dogs and cats were registered in 40.5 %. Ringworm in cats often manifest as asymptomatic disease in the form of a prolonged ecdysis. In dogs with a thick coat (Chow-Chow, German, Caucasian Shepherd Dog, outbreed, etc.), and also with well-developed subcutaneous tissue (Rottweilers, American Bulldogs, Shar Peis) has often noted the ringworm, as limited moist areas. In dogs with short hard hair (Dachshund, American Staffordshire Terrier, French Bulldog, Great Dane, etc.) have noted an inflammatory response of varying severity; itching from easy to exhausting, scratching, formation of crusts. Lack of movement, non-balanced ration in vitamins and mineral substances, inbreeding, groundless taking of antibacterial, vitamin and other preparations cause the decrease of skin protection functions. Dermatites, which are treated with difficulty, are the result of functional disorders of excretory system, digestive system and also glands of internal excretion which weaken animal organism and destroy processes of epidermis regeneration. All above mentioned make favorable conditions for existence of microscopic fungi.

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SEROPREVALENCE OF *YERSINIA ENTEROCOLITICA* SEROVAR O:9 IN FARM ANIMALS

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Summary. Intestinal yersiniosis is a dangerous infectious disease of farm animals caused by *Yersinia enterocolitica*. In young animals the disease occurs with symptoms of gastro-intestinal tract lesions. The subclinical form of the disease is detected in adult animals. Infected animals remain agents during the whole period of the life and there are a threat for livestock and human health. Such animals are identified by serological studies. Nowadays the research on the distribution of intestinal yersiniosis in Ukraine has been conducted insufficiently.

The results of the conducted serological screening of intestinal yersiniosis causative agent of *Yersinia enterocolitica* serovar O:9 on the animal-breeding farms of the different regions of Ukraine. Serological screening was done on the 15 farms in 7 regions of Ukraine, 213 blood serum samples from cattle, sheep, pigs were examined.

Keywords: *Yersinia enterocolitica*, serovar O:9, serological screening, seroprevalence, farm animals

Introduction. Intestinal yersiniosis is an acute contagious disease of many species of animals and human. The disease is characterized by lesions of gastro-intestinal tract, organs of respiratory system, arthritis, pyo septicemia. The course of the disease in adult animals is mainly chronic, resulting in a depletion and inhibition of body weight augmentation. The acute course of the disease causes abortions, the birth of non-viable young animals, infertility. The long duration bacteriocarrier is widely spread. The intestinal forms of the disease is detected young animal. This form of disease has an acute and subacute stages with the symptoms with diarrhea and the suppression of nervous system. The mortality can be 50%. This disease occurs worldwide (Jamali, Radmehr, and Ismail, 2014; Laukkanen-Ninios et al., 2014; Liang et al., 2012). Infection has been identified in animals in Europe, the USA, South America and Asia (Capita et al., 2002; Schaake et al., 2013). As in our country, as abroad intestinal yersiniosis is widely spread and takes an important role in animal and human pathology(Liang et al., 2012; MacDonald et al., 2011). The problem of Yersiniosis became topical because morbidity rate among people had increased (Rahman et al., 2011). According to data of the World Health Organization, Yersiniosis is registered in more than 30 countries around the World and it occupies the 4th place in the structure of zoonotic infections, about which would be reported. In European Union intestinal yersiniosis spread is 1.92 cases per 100,000 population (EFSA and ECDC, 2015). In spite of epizootological and epidemiological significance of Yersiniosis today in Ukraine research on the spread of intestinal yersiniosis held at insufficient.

The non-correspondence to sanitary rules during provision, transport and storage of foodstuff causes their contamination with pathogen and leads to infection of animals (Bolton, Ivory, and McDowell, 2013). The disease manifests itself in some animals sporadically or as enzootic outbreak. The economic importance of this disease is arise not only from the death of young animals, and above all of the losses related to the inhibition of body weight augmentation, decreased reproductive efficiency and growth retardation (Rahman, et al., 2011). Infected animals remain lifelong carriers and pose a threat for livestock and human health (Liang et al., 2012). Clinical examination of animals and serological investigation of blood sera samples conduct to establish the preliminary diagnosis.

The goal of the researcher work is conduction of serological screening for *Yersinia enterocolitica* serovar O:9 in ruminants and pigs in Ukraine.

Materials and methods. For conduction of serological screening there were collected blood sera of adult mature animals and examined its by serum agglutination test in tube.

Blood serum samples were examined in adult mature animals (cattle, sheep and pigs) from productive and breeding farms. All observed animals had no clinical signs of disease.

There were collected 213 blood serum samples from cattle, 86 blood serum samples from sheep, 36 blood serum samples from pigs. Total number of examined blood serum samples was 335.

Serological examination was used by SAT (serum agglutination test in tube). Blood serum samples were examined by using the tube agglutination test with antigen *Yersinia enterocolitica* serovar O:9 (TC U 46.15.091-95). Serum dilution varied from 1:50 to 1:600.

Results. Serological monitoring of intestinal yersiniosis was conducted from 15 farms in Central, Eastern and Southern regions of Ukraine. There were examined 335 samples of blood serum of farm animals. Blood serum samples were collected from 6 cattle, 2 sheep, 7 pig farms (Table 1).

Table 1 — Results of study of blood serum samples by SAT with *Yersinia enterocolitica* diagnosticum

№	Farms	Number of blood serum samples	Assessment of results		
			positive	doubtful	negative
Cattle					
1	Farm № 1, Kirovograd region	10	1	2	7
2	Farm № 2, Cherkasy region	10	8	1	1
3	Farm № 3, Kharkiv region	11	1	3	7
4	Farm № 4, Kharkiv region	113	20	29	64
5	Farm № 5, Kharkiv region	67	7	23	37
6	Farm № 6, Kharkiv region	2	—	2	—
Total		213	37	60	116
Sheep					
1	Farm № 1, Kharkiv region	52	3	9	40
2	Farm № 2, Kharkiv region	34	—	—	34
Total		86	3	9	74
Swine					
1	Farm № 1, Dnipropetrovsk region	5	2	1	2
2	Farm № 2, Odessa region	6	—	—	6
3	Farm № 3, Kharkiv region	5	—	—	5
4	Farm № 4, Donetsk region	4	—	—	4
5	Farm № 5, Kirovograd region	6	—	—	6
6	Farm № 6, Sumy region	2	—	—	2
7	Farm № 7, Odessa region	8	—	—	8
Total		36	2	1	36

High level of seroprevalence from 70% to 90% was detected in all examined groups of ruminants animals. When examining animals the high level of seroprevalence was registered in 92.02% of the cattle and in 75.86% of sheep. However, most of them had the titers that were lower than the diagnostic level. Such results are taken into consideration us doubtful positive once.

During study high seroprevalence were found in 6 cattle farms. Thus, 71.83% of samples was positive. 17.37% of samples had high diagnostic titers (1:200 and above), the other 54.46% of samples had 1:50 and 1:100 titers, what means 'doubtful positive' result (Fig. 1).

Similar trend was seen after examining of 86 sera samples of 2 sheep farms of Kharkiv region. Seroprevalence rate reached 89.54%. However, diagnostic titers were detected only in 3.49% of animals, the other 86.05% was 'doubtful positive' results (Fig. 2).

As for the pigs 36 animals were examined and small numbers of seropositive animals were revealed — 8.32%. When examining the pigs, diagnostic titers were in 5.5% of animals, 2.77% showed 'doubtful positive' results (Fig. 3).

One sample showed the result for ++++ in the titers 1:400, that can testify the acute course of the disease. The conducted serological screening of intestinal yersiniosis had proved high level of seroprevalence in farm animals (Fig. 4).

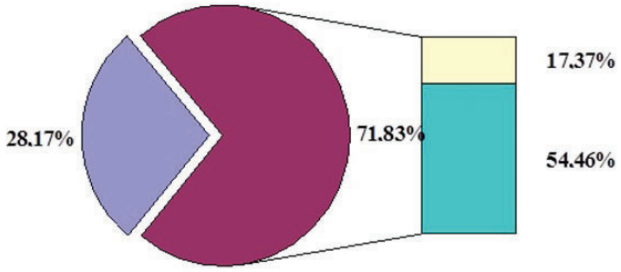

Figure 1. Seroprevalence of *Yersinia enterocolitica* serovar O:9 in cattle

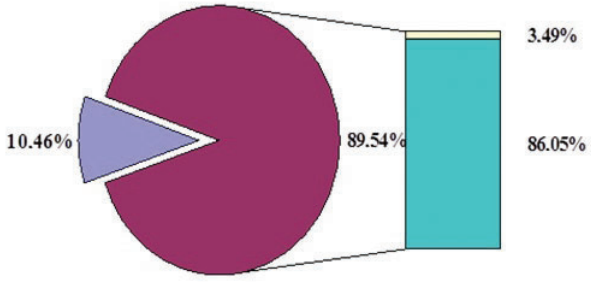

Figure 2. Seroprevalence of *Yersinia enterocolitica* serovar O:9 in sheep

The conducted serological investigation of intestinal yersiniosis has proved high level of seroprevalence in farm animals.

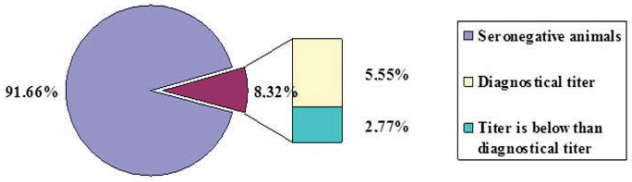

Figure 3. Seroprevalence of Yersinia enterocolitica serovar O:9 in pigs

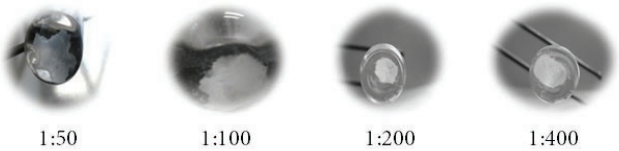

Figure 4. The investigated sample of blood serum pig

Conclusions. According to the results of the research the presence of high level of seroprevalence of *Yersinia enterocolitica* in farm animals confirms asymptomatic bacterial carrier status in industrial herds of cattle, sheep and pigs.

The presence of 'doubtful positive' reactions talk about circulation of low virulence *Yersinia* isolates, which in case of reversion may pose a threat for animals and people health.

Thus results of our research demonstrate topicality of intestinal yersiniosis monitoring for realization of control and prevention of the disease in agricultural farms of Ukraine.

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Part 2. Biosafety

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RISK ANALYSIS AND MOLECULAR EPIDEMIOLOGY ASPECTS FOR EMERGENT DISEASES OF ANIMALS (AFRICAN SWINE FEVER, BRUCELLOSIS, AVIAN INFLUENZA AND NEWCASTLE DISEASE)

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Summary. The risk analysis is the one of crucial points of the modern approach for the epizootic situation assessment and biorisks management system. Most important for epizootic situation control and international trade management is the system of transboundary diseases distribution risk determination. This allows creating forecasts regarding diseases introduction and distribution among the countries and territories and to develop appropriate strategies of the diseases control and minimize trade-associated diseases related risks. The paper involves description of the basic definitions in area of the risk assessment, strategies and approaches for its determination and analysis. The role of the molecular genetics tools are described from the position of effectiveness for risks forecasting in wildlife and animal husbandry.

Keywords: risk analysis, molecular epidemiology, emergent diseases, animals

The globalization of the modern world, increasing volumes of international trade operations, transport people, animals, agricultural products considerably exacerbates the risk of occurrence and spread of infectious diseases, as well as the distribution of pathogens that cause them. This explains the fact that the biological safety is a key component of national security.

The issues of biosafety in the world dealing with international organizations: World Health Organization (WHO), the Office International des Epizooties — World Organisation for Animal Health (OIE) and the World Organization for Agriculture and Food Organization (FAO).

Adopted by the WHO, OIE and FAO concept 'One health' (One Health — health care consumers by producers and product safety), which is currently sold as a common strategy that consolidates effort veterinary and humane medicine in the areas of assessment and management of biological and nutritional risks resulting from the emergence and spread of infectious diseases, disorders of feeding, animal keeping, processing and quality control and safety of agricultural products.

The main objective of veterinary and humane medicine in the context of the implementation of Biosecurity is to identify and minimize biological ***threats*** (***hazards***) and risks associated with infectious diseases and their agents.

Risk is the potential situation of losing or gaining of something (Kungwani, 2014). The ***risk*** could be also determined as the likelihood that harm will occur. The definition of the risk is strongly aligned with the term hazard — something that has the potential to do harm. The minimization of the possible harm effect is associated with risk management.

Most crucial of the effective risk management is the assessment of risks, that includes the determination of quantitative or qualitative estimation of risk related to a well-defined situation and a recognized hazard (threat).

An acceptable risk is a risk that is understood and tolerated usually because the cost or difficulty of implementing an effective countermeasure for the associated vulnerability exceeds the expectation of losses.

Most risks in veterinary medicine concerning animal health and agricultural products quality and safety are associated with infectious diseases. These are the most crucial and require complex control and prevention. Especially in cases of the emergent and zoonotic diseases, associated with possibility and probability of affection of public health and economical situation in countries and regions.

The practices of the biorisk assessment are based on the analysis of risks for potential introduction of the diseases, analysis of the outbreaks occasion risks, analysis of possible affects and the summarization of the biorisks assessment.

The international approach for the risk analysis data collection and use includes determining of the country/territory status in order to:

— identification of the opportunities for transactions of import and export of agricultural products;

— analysis of global epizootic situation on cross-border and other emergent diseases (African swine fever, influenza, Newcastle disease, bluetongue, Shmallenberg virus, foot and mouth disease, etc.);

— control of the 'program diseases' (MDR-TB infections, bovine leukemia (in the case of Ukraine), etc.).

Control of diseases in the regions is aimed to protect national export status, that includes identification of the surveillance strategy, analysis of internal (including human-associated) risks, and identification of the prevention strategy.

The legislative background of the biological and epizootical risks assessment internationally is based on three main key points of legal documents (Sundqvist et al., 2013):

1. Legal documents of the World Trade Organization (WTO) includes definition of regions with low prevalence infectious diseases for the purpose of trade operations: Article 5 'Assessment of Risk and Determination of the Appropriate Level of Sanitary or Phytosanitary Protection' and Article 6 'Adaptation to Regional Conditions, Including Pest- or Disease-Free Areas and Areas of Low Pest or Disease Prevalence' of the Agreement on the Application of Sanitary and Phytosanitary Measures (WTO, 1995);

2. Manuals and Codes of the Office International des Epizooties — World Organisation for Animal Health (OIE), standards for international trade in animals and animal products;

3. National regulations (e.g., for Ukraine):

— the Law of Ukraine 'On veterinary medicine';

— veterinary legislation;

— ISO, DSTU, and SOU guidelines for the monitoring and diagnosis of infectious animal diseases.

One of the main principals used for field/regional risk assessment in the veterinary field is the principal of the regionalization.

Following a formal request from the importing or exporting country, the following steps are executing in this process, including familiarization with primary information about the country/territory, detection of lack of information and request additional data, visit the site to be tested, determining the risks of imports, the status of 'free' area of disease/pathogens, transport/migration routes for risk control, written report on risk assessment preparation, and its sharing, forming of regionalization policy.

The system of the epizootological wellbeing/public health involves multiple approaches, including detailed description of the region and livestock in it, characterization of the region borders, compartmentalization regions and territories, marking of the major and minor settlements, roads, transportation and others, communications, location of the district veterinary service, official laboratory, border guards and transport, other important bodies responsible for the maintenance of livestock, identification of international and inter-regional trade ties, and, finally, specification diseases are risk factors.

The typical risk assessment matrix is demonstrated on the Fig. 1, where the risk category could be ranged from low to extremely high, depending from the probability and severity of the risk.

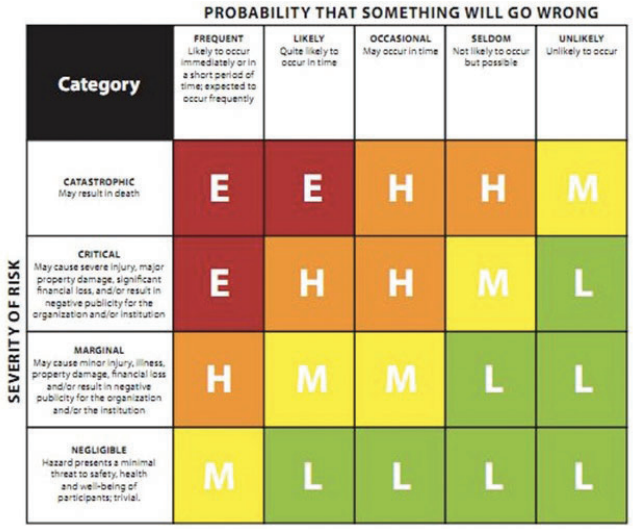


Figure 1. Risk assessment matrix (from https://q9cqualityconsulting.com/2011/09/22/risk-probability-can-we-do-better/comment-page-1/)

The regional risks estimation is passed on another plot, which involves multiple factors of risk, and its possibility and probability. The risk possibility score calculated by the points. The high possibility is estimated as 3 points, low — 1 point, and rare — 2 points. Assumed scores could be subdivided to 5–7 categories of risks from the low to extremely high (Table 1).

Table 1 – Transboundary risks estimation matrix

Risk factor	High level	Midlevel	Low level
Existing of the disease in the wildlife	?	?	?
Existing of the disease in domestic animals	?	?	?

Existing of the transmission vectors	?	?	?
Existing of the transboundary transmission ways (migratory wildlife)	?	?	?
Transport communications (migration of people and trade)	?	?	5–7 risk categories

African swine fever. Illustration of this risk assessment model could be described on the example of African swine fever (ASF). ASF was reported in Ghana, Nigeria, Lithuania, Latvia, Belarus and Poland and the Russian Federation in 2015 (2007 in Russia recorded more than 450 outbreaks of disease among domestic pigs and 200 cases among wild boars) (Fig. 2).

In accordance with the EU Directive 2016/464 with ASF were affected multiple EU regions, including: Estonia (28 affected regions), Latvia (16 regions), Lithuania (11 regions), Poland (14 regions, 48 points). In the mentioned regions held zoning and implemented quarantine measures. The Supervisory zone is also referred about Sardinia (Italy), where ASF outbreak was recorded among wild and among domestic pigs (EC, 2016).

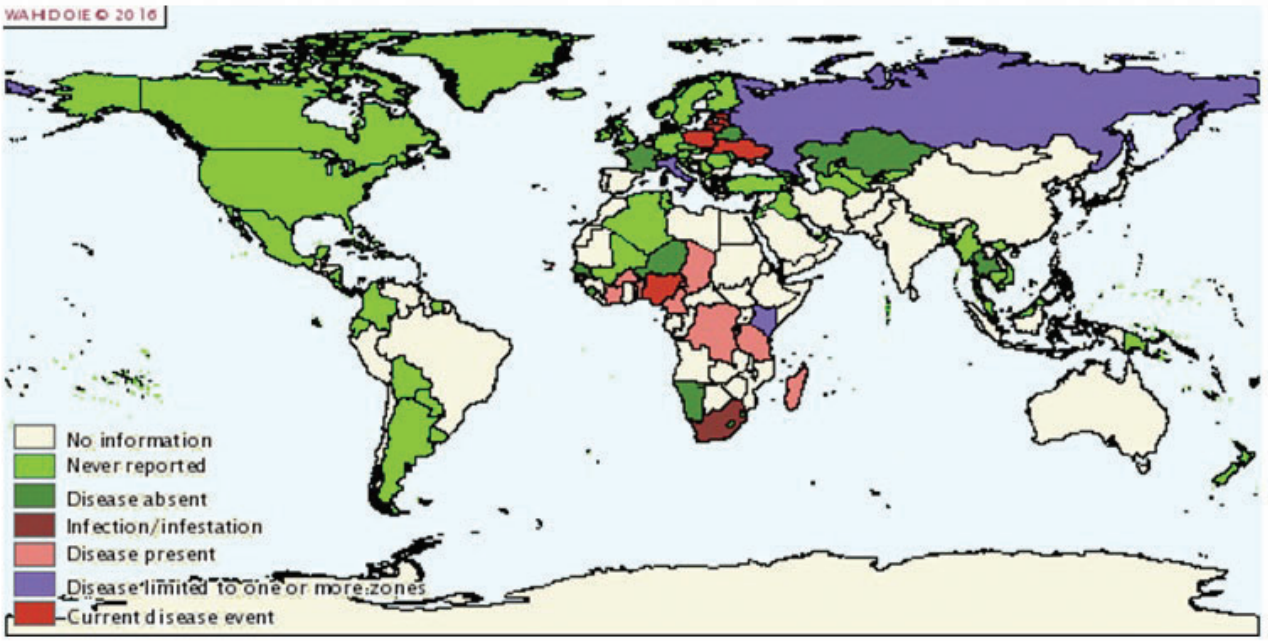


Figure 2. Epizootic situation for the ASF in the World (from http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap)

The genetic relationship of the virus indicates the presences of 22 genotypes are roughly divided into two genetic lineages. The marker genotype-specific area is the p72-region. The progenitors of the first genetic line are clusters of pathogens from genotype I, which circulated in Africa and Europe in 50–60 years, and the second — 8 genotype isolates Caribbean in 70s.

Today on the territory of Kenya and Uganda, according to the monitoring of populations of ticks and warthog circulating virus genotypes IX and X.

The 'molecular watch' demonstrates development of the 'modern' 2nd genotype population progenitor-relation from the old African population of the virus. This agent population was introduced via Mideast to Caucasus region, affected Georgia, Armenia, Azerbaijan and Russia. After that in has been introduced in Ukraine (2012), Belarus (2014), and other countries (Fig. 3) (Gallardo et al., 2015).

The molecular tools used for the characterization of the ASF agent demonstrate similarity among isolates that proves its transboundary dissemination between countries: from Georgia and Armenia to Azerbaijan and Russia, and from Russia to Ukraine, Belarus. These three countries are the partitions of the way of ASF distribution to Poland and Baltic states (Fig. 4) (Gallardo et al., 2014).

Using of the epizootical statistics data and previously described risks assessment matrix we can calculate the transboundary introduction risks for re-introduction of ASF virus in Ukraine from the Eastern border (Table 2). Calculation of the summary of risk points (n = 14) the risk could be characterized as extremely high confirmed risk.

The correlation among molecular characteristics and relative homology of virus circulating in neighbor countries could be the confirmation of risk existence and source of disease introduction.

Table 2 – Transboundary risks estimation for ASF introduction from Russia for Eastern Ukraine

Risk factor	High level	Midlevel	Low level
Existing of the disease in the wildlife	+		
Existing of the disease in domestic animals	+		
Existing of the transmission vectors	?		
Existing of the transboundary transmission ways (migratory wildlife)	+		
Transport communications (migration of people and trade)		+	3 + 3 + 2 + + 3 + 2 = 14 Extreme risk (confirmed)

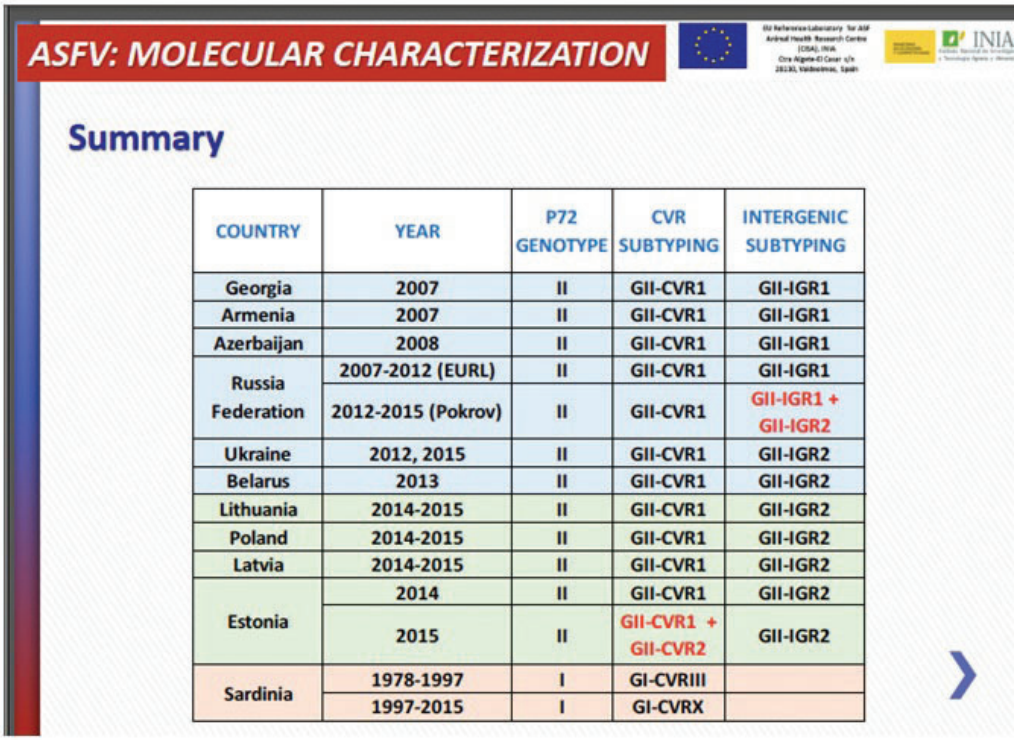
Brucellosis. Brucellosis is especially contagious infectious zoonotic disease that affects different types of animals and people. Its agent belongs to the genus *Brucella*, which includes 9 species. The most severe forms of the disease causing by *Brucella melitensis, Brucella abortus,* and *Brucella suis*. Brucellosis occurs in acute or chronic forms of especially dangerous epizootic and epidemiological point of view is latent *Brucella*-keeping. Patients affected the reproductive organs of animals, at least — other organs and tissues. Economic losses caused by brucellosis in the mass abortion, birth of weak calves and non-viable, stable formation of infertility in adult animals (OIE, 2016).

The source of pathogen infection is the sick animals and *Brucella*-carrier. The reservoir of the pathogen is represented by wildlife (wolves, hares, wild boar, elk, deer), pets (dogs, cats), synanthropic rodents and birds, as well as blood-sucking insects and mites.

Factors of transmission are abortion-foetus, exudates and isolating sick animals, milk, contaminated genetic resources (semen, embryos), livestock products, feed, water, manure or litter, veterinary equipment and instruments.

Brucellosis is transmitted alimentary, contact, sexual and ways aerogenic (Stegniy et al., 2015).

Figure 3. Molecular profile of ASF in Eurasia (from http://asf-referencelab.info/asf/images/files/WS%20CISA%202014/PPTS/08_Arias_M__ASF_Gen_Activ_URL_2014.pdf)



COUNTRY	YEAR	P72 GENOTYPE	CVR SUBTYPING	INTERGENIC SUBTYPING
Georgia	2007	II	GII-CVR1	GII-IGR1
Armenia	2007	II	GII-CVR1	GII-IGR1
Azerbaijan	2008	II	GII-CVR1	GII-IGR1
Russia Federation	2007-2012 (EURL)	II	GII-CVR1	GII-IGR1
	2012-2015 (Pokrov)	II	GII-CVR1	GII-IGR1 + GII-IGR2
Ukraine	2012, 2015	II	GII-CVR1	GII-IGR2
Belarus	2013	II	GII-CVR1	GII-IGR2
Lithuania	2014-2015	II	GII-CVR1	GII-IGR2
Poland	2014-2015	II	GII-CVR1	GII-IGR2
Latvia	2014-2015	II	GII-CVR1	GII-IGR2
Estonia	2014	II	GII-CVR1	GII-IGR2
	2015	II	GII-CVR1 + GII-CVR2	GII-IGR2
Sardinia	1978-1997	I	GI-CVRIII	
	1997-2015	I	GI-CVRX	

Figure 4. Molecular characteristics of ASF virus, allocated in European region (from http://www.efsa.europa.eu/sites/default/files/151123-p7.pdf)

Scientists of NSC 'IECVM' played a key role in the scientific support of the eradication of brucellosis, bovine brucellosis has been completely eradicated in Ukraine in 1975 against the backdrop of its widely spread in Russia, Romania, Hungary and Kazakhstan (Obukhovska et al., 2014).

Today there is a risk of transboundary animal brucellosis entry to Ukraine from countries disadvantaged on the infection (including Russia, Georgia, Turkey, Greece, Serbia, Iran, Mongolia, China) via:

— while export-import operations (via infected breeding or productive animals, animal products or genetic resources);

— as a result of a possible cross-border migration of wildlife.

The surveillance and diagnostics tools were developed in NSC 'IECVM' for the brucellosis control in Ukraine:

— national standard of Ukraine — anti-*Brucella abortus* serum;

— positive and negative control sera set for AT and CFT;

— serological kit for differentiation of *Brucella* cultures,

— RS-antigen for AT,

— serological test kits for diagnosis of infectious epididymitis in rams LCFT and AGID.

To ensure the scientific support of production of biological products for the diagnosis and prevention of brucellosis in the NSC 'IECVM' created the unique Ukrainian collection of *Brucella* strains (135 strains), including:

- 4 strains of *B. melitensis*;
- 71 strain of *B. abortus*;
- 15 strains of *B. suis*;
- 45 strains of *B. ovis*.

The studies of the molecular genetic characteristics of strains collection are performed using PCR, restriction analysis and sequencing of the genome.

The perspective studies for risk assessment concerning the animal brucellosis are: monitoring in the wild (especially in the areas of risk of transboundary drift illness), and molecular genetic characterization of *Brucella* collection.

Avian influenza and Newcastle disease. The viruses that cause highly pathogenic avian influenza (HPAI) and Newcastle disease (ND) are currently eradicated in poultry in Ukraine. But the often-occurred in EU and Russia, so could be potentially transmitted via wild birds. Especially significance of these diseases demonstrates recent outbreaks of H5N8 in Germany, Italy and other member states of EU. However, the zoonotic threat of their spillover and emergence into poultry remains due to the presence of infected wild birds. Emergence is further supported by the viruses' intrinsic genetic and antigenic variability, which can facilitate host jumping and switching. In addition, global warming and climate change may increase viral transmission among reservoir and non-reservoir hosts (Gerilovych et al., 2008;

Stegniy, Gerilovych, and Stegniy, 2008; Gerilovych and Potkonjak, 2009; Muzyka et al., 2014).

Historically, these diseases natural nosology areas were concentrated in Southern East Asia, but since last years it was enlarged (Fig. 5), and the new subtype of the highly pathogenic avian influenza virus, H5N8 has been occurred. It distributed in Americas, Africa, and Eurasia. Taking into account the molecular epizootology profile of HPAI in 2005/2006 and 2008, when the origin of virus was primary from Russia, and next time from EU countries (Fig. 6), and the non-wellbeing status of big amount of EU and CIS countries, the risks of new type introduction in poultry of Ukraine is extremely high. These risks are associated with wild birds, migrating via territory of our state.

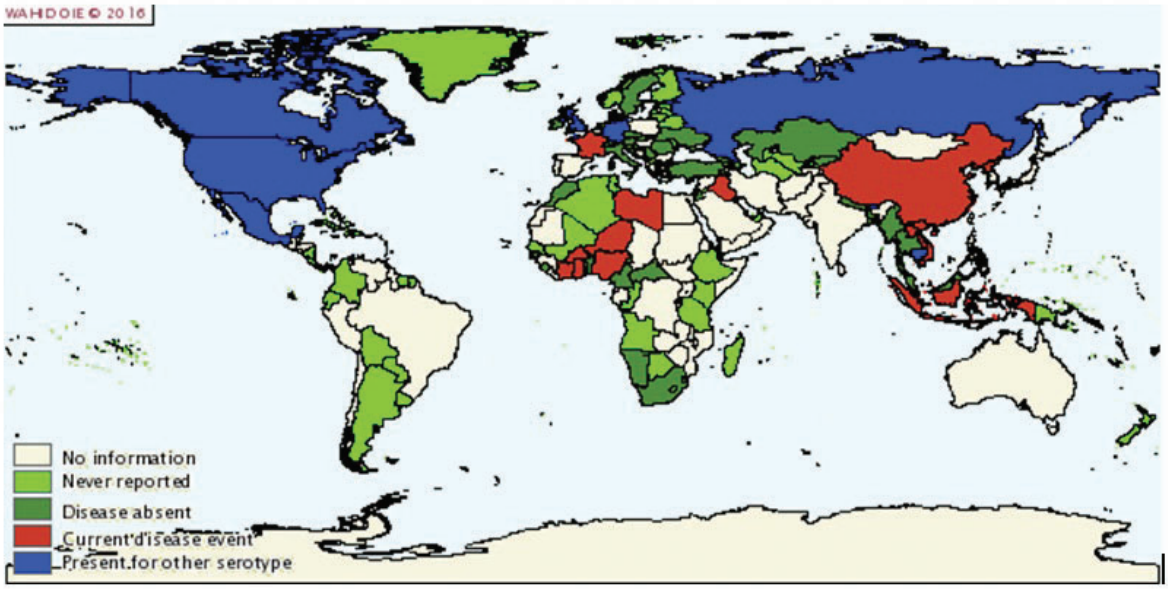


Figure 5. New HPAI epizootic situation (from http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap)

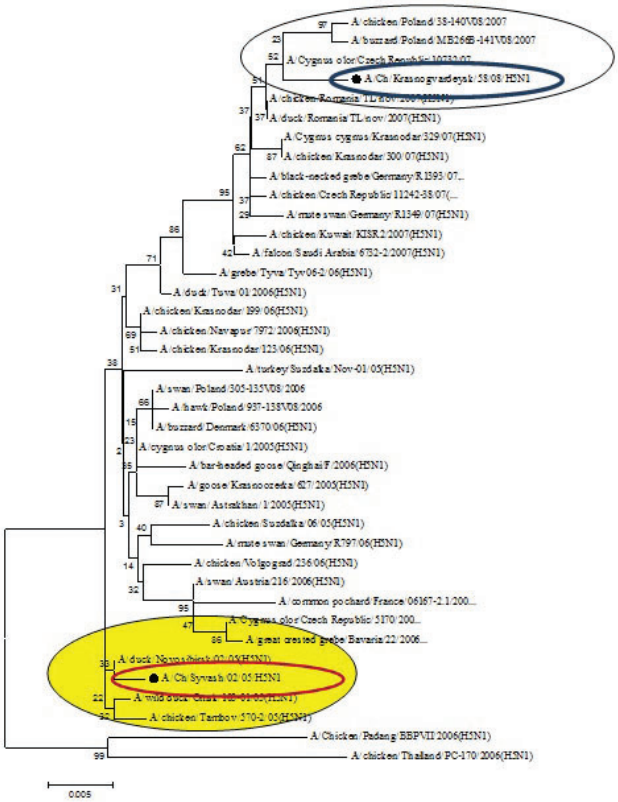


Figure 6. Phylogenetic relations of Ukrainian isolates (•) of HPAI with EU and Russian isolates

World epizootic situation concerning Newcastle disease also demonstrates, that disease endemic area from Southern-East Asia was moved to African countries (Fig. 7).

The molecular epizoothology data collected by NSC 'IECVM' demonstrate circulation of 1st (historically), 2nd, 4th, and 5th genotypes (according Aldous et al., 2003) of NDV in Ukraine. These viruses have Western European, Eastern European and Asian origin (Fig. 8).

Migratory crossways of the wild birds can introduce 'African' viruses to our state. Potential of this action was demonstrated in our previous study concerning other paramyxoviruses. Also, as potential harm of Newcastle disease virus introduction could be determined Romania.

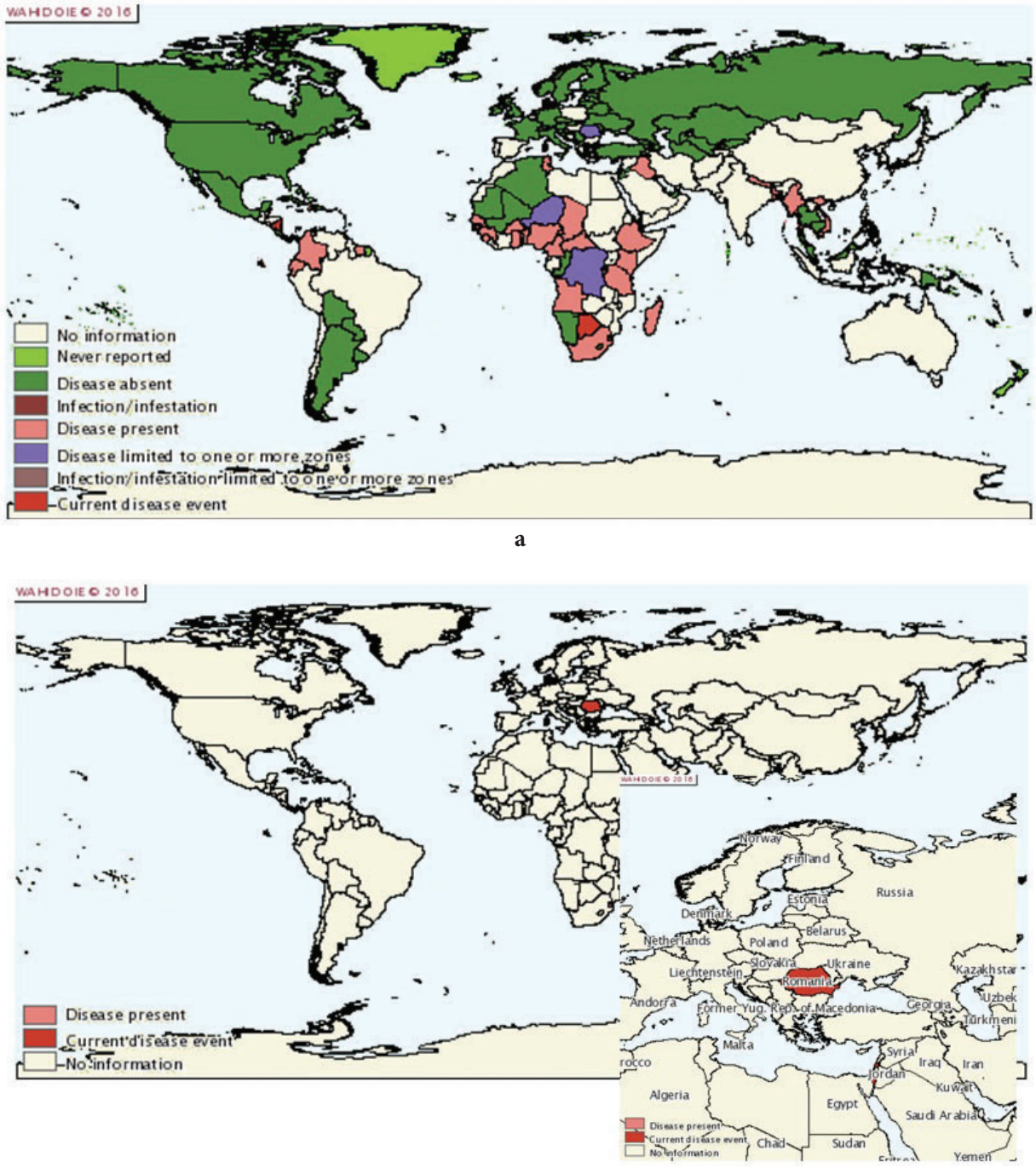


Figure 7. Epizootic situation concerning Newcastle disease in the World and EU countries: a — 2015, b — 2016) (from http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap)

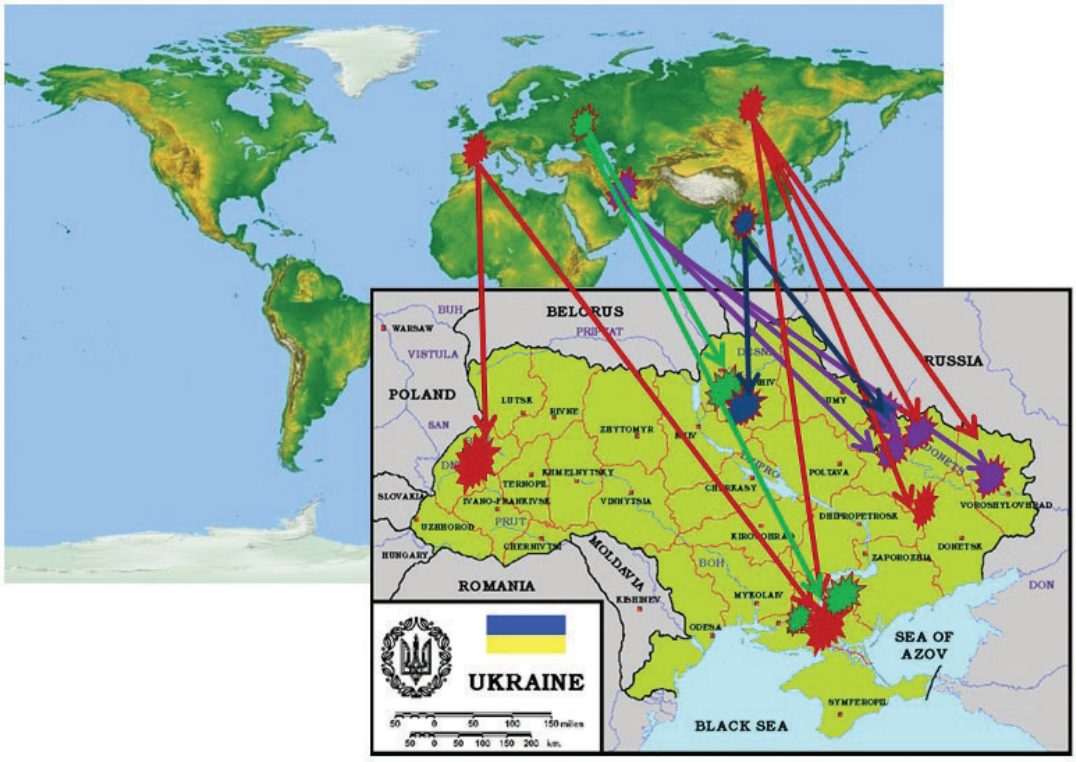


Figure 8. Origin of Ukrainian strains of Newcastle diseases from different genotypes

Conclusion. The epizootical risks for emergent diseases transboundary introduction could be estimated by the complex risk factor assessment. The existing risk could be ranged to 5–7 categories of risks, including low, moderate, and high clusters. The extremely high risks of ASF virus introduction are existed for Eastern Ukraine from Russia. Moderate to high-level risk of transboundary animal brucellosis entry to Ukraine from disadvantaged on the infection of countries (including Russia, Georgia, Turkey, Greece, Serbia, Iran, Mongolia, China) exists. High-level risks are estimated concerning avian especially dangerous pathogens introduction to Ukraine via migratory birds. Using of the molecular genetics tools allow to determine viruses and bacteria host and geographical origin. This could be used in identification of the risk source and let enhancing of the existing surveillance with more target capacity to develop effective countermeasures against emergent animal diseases.

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UNEARTHING ANTHRAX — APPLICATION OF GENOTYPING FOR EXPLORING A CRYPTIC LIFE CYCLE OF *BACILLUS ANTHRACIS* IN SOIL

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Summary. *Bacillus anthracis*, the etiological agent of the zoonotic disease anthrax is a very monomorphic species. Typically, the epidemiology of anthrax outbreaks is investigated employing progressive hierarchical resolving assays using nucleic acids (PHRANA). For resolution of relationships of *B. anthracis* originating from a single animal this approach is not suited. In order to close this gap PHRANA can be amended with whole genome sequencing data and subsequent analysis of Single Nucleotide Polymorphisms (SNPs). Doing so, it was possible to resolve the genetic diversity of isolates from anthrax outbreaks in Sweden, Italy as well as of drug use-related infections in Europe. The Swedish outbreak was confined to a short time-period whereas the Italian anthrax foci were revisited for analysis ten years after the host animals have died. Data from the study in Italy contrast with the established view concerning a strict resting stage of *B. anthracis* in soil. This review discusses the plausibility that *B. anthracis* multiplies in a limited soil-borne life cycle after the spores have diffused to near the surface where the bacteria encounter favorable conditions in non-animal hosts or rhizosphere.

Keywords: *Bacillus anthracis*, anthrax, soil, genotyping, life cycle

Evidence that *B. anthracis* is not fully inert while residing as a spore in soil. Pathogenesis of *B. anthracis* infections in animals and man is well understood and the pathogen's virulence factors are studied in great detail (Turnbull, 2008). Much less is known about the genetic diversity of the bacterium within a single infected animal or within different sick animals of the same herd. All but nothing is known about the period after the bacterium has killed its host and sporulation has been completed. What happens after *B. anthracis* has entered the part of its life cycle known as endospore in the environment, e.g., soil, must be considered a black box. What is known is that endospores can endure for decades in soil constituting the natural reservoir of *B. anthracis* (Hugh-Jones and Blackburn, 2009). These sites of contamination pose a high risk of reinfection for grazing animals presumably either via inhalation or ingestion of endospores. However, outbreaks occur only sporadically with time intervals ranging from years to decades and typically these outbreaks are seasonal following a period of hot-dry weather and rain fall (Turnbull, 2008).

Despite the prevailing theory that in nature *B. anthracis* is an obligate pathogen restricted to a metabolically inactive endospore state outside the host, an early hypothesis postulated multiplication of *B. anthracis* in 'incubator areas' (soils rich in organic matter and calcium with a pH above 6.0 and an ambient temperature above 15.5 °C) (Van Ness, 1971). The sporadic occurrence of anthrax outbreaks under certain climatic and environmental circumstances could then be explained by local accumulations of *B. anthracis* that reach the required dose for infection of grazing animals due to multiplication in near-surface soil. A competing hypothesis suggests that these local accumulations emerge from the physical pooling of endospores in rainwater depressions because of the endospores' hydrophobic surface character. Furthermore, vegetative cells of *B. anthracis* were suggested to be unable to successfully compete with resident soil microbiota (Sterne, 1959) and have never been found in the environment. Also, the clonal genetic character of the organism argues against frequent episodes of soil proliferation. Thus, the lifestyle of *B. anthracis* in the environment has jokingly been summarized as 'sporulate or die' (Turnbull, 2008).

An increasing amount of laboratory findings, however, contrast with this established view of an obligate pathogen. Since other members of the genetically homogenous *B. cereus* sensu lato group was found to multiply in guts of soil associated invertebrates, as a saprophyte in the rhizosphere of plants and also in sterilized soil, it would be surprising if multiplication of *B. anthracis* is strictly limited to an animal host body. Indeed, repeated probing of a natural focus in Namibia at which an animal had died from anthrax, showed that *B. anthracis* spore counts increased around grass roots over the years (Saile and Koehler, 2006) indicating multiplication in the rhizosphere which could already be observed under laboratory conditions. Later work strongly suggested the proliferation of *B. anthracis* within soil-dwelling amoebae (Dey, Hoffman, and Glomski, 2012). Additional experiments showed that lysogeny of *B. anthracis* with bacteriophages may enable the bacterium the colonization of the intestinal tract of earthworms (Schuch and Fischetti, 2009). These findings suggest that *B. anthracis* is principally

competent to grow in suitable soil environments, but this has not been observed in the environment thus far. However, frequent probing of contaminated soils over several years revealed isolates of *B. anthracis* lacking one or both virulence plasmids (Antwerpen et al., 2011; Aikembayev et al., 2010). This loss, on the background of the aforementioned laboratory findings, can be explained most simply by a soil-borne life cycle of *B. anthracis*. Recent work conducted as part of a collaborative project between partners from Germany and Italy has explored using genetic methodologies the possibility of a cryptic life cycle in soil. The results and conclusions are outlined in the next section.

***B. anthracis* retrieved from aged anthrax foci appears to have undergone some degree of microevolution.** Although occurring only sporadically, anthrax is endemic in southern Italy. The most severe epidemic during the last decade started in 2004 in the region of Basilicata with a total of 124 animals, mostly cattle but also sheep, goats, horses and red deer (Fasanella et al., 2010). In 2014, sites were revisited where in 2004 cows diseased from anthrax had been buried. From two of these aged foci samples were drawn (Fig. 1) to test the hypothesis of a soil-borne life cycle (Braun et al., 2015). The rationale of this work was if there was *B. anthracis*-proliferation then genotypes of strains isolated from near the surface (5 cm) of contaminated soil should be on a different evolutionary trajectory from those residing at 100 cm depth near the bovine carcass. It was the expectation that the surface population would yield a higher genetic diversity and such possible microevolution was evaluated using genomic tools.

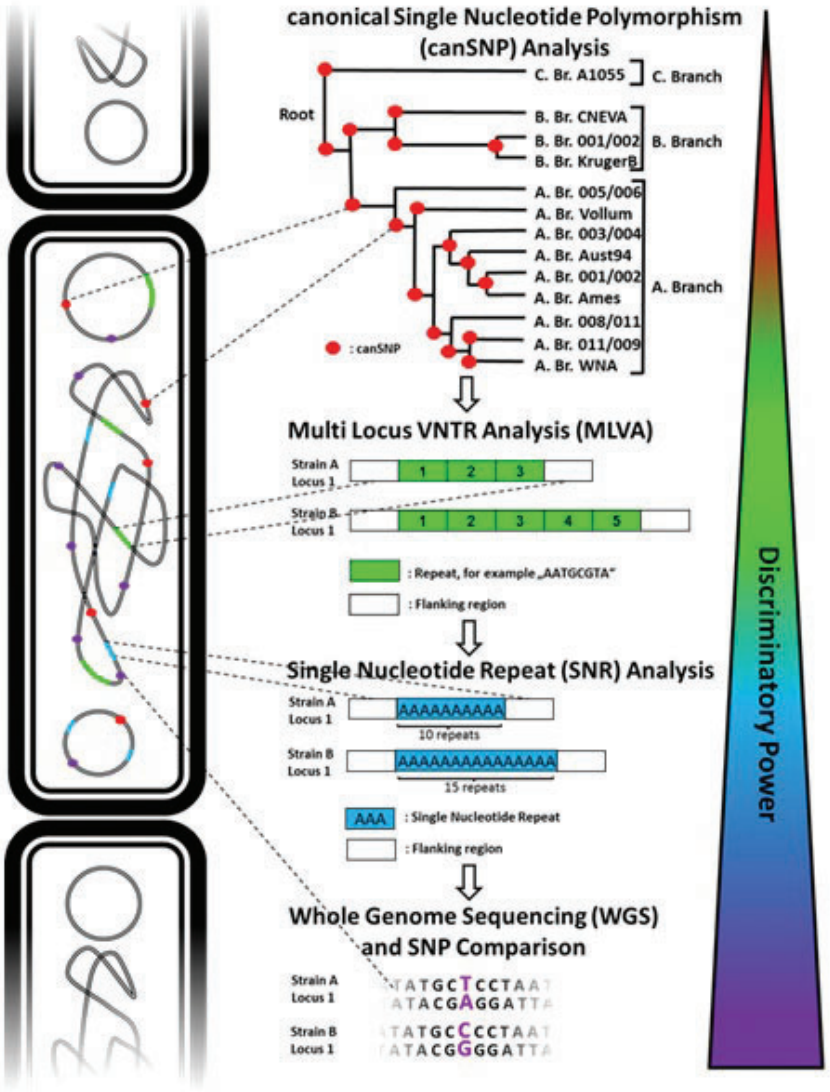

Figure 1. Unearthing *Bacillus anthracis*. Left Panel: Drilling core of a hand-held ground auger with a soil-sample from an anthrax burial site at Pollino National Park (Italy). Right panel: Agar plate after processing such a soil sample using the GABRI method (Fasanella et al., 2013). Non-hemolytic colonies of *B. anthracis*, which appeared whitish and medusa-head shaped, can be easily identified and distinguished from contaminants

B. anthracis can be a challenging organism for genetic analysis in terms of differentiating the species from its close relatives of the *B. cereus* sensu lato group. Conversely, on the genomic level, *B. anthracis* itself is a very clonal organism with little if at all horizontal gene transfer. Evolution is considered to be restricted to the limited reproduction phases of 20–40 generations during host infection while the ensuing endospores may lay dormant for years away from any host (Keim et al., 2004). Since its rather recent emergence as a pathogen about 3,000–6,000 years ago (Van Ert et al., 2007) only a small degree of genetic variations is observed when comparing strains from different parts of the world. Analysis of whole genome data revealed variations mainly in Variable Number of Tandem Repeats (VNTR), Single Nucleotide Repeats (SNR) and Single Nucleotide Polymorphisms (SNP). A subset of the SNPs is representative for the three major lineages A, B and C of *B. anthracis* which can be further divided into thirteen different original canonical SNP (canSNP) branches that reflect the global phylogenetic relationships among *B. anthracis* strains (Van Ert et al., 2007). Within a single distinct canSNP group, Multi Locus VNTR Analysis (MLVA) can be used to further differentiate strains, e.g., in outbreak investigations because of the greater diversity values within these markers due to higher mutation rates in VNTRs compared to point mutations (Keim et al., 2004; Van Ert et al., 2007). Finally, analysis of highly mutable SNR markers serves as a tool to discriminate between very closely related isolates even within a single MLVA genotype during enzootics (Keim et al., 2004). This hierarchical fingerprinting system is called PHRANA (Progressive Hierarchical Resolving Assays using Nucleic Acids) and is commonly used for epidemiological investigation of outbreaks as well as for trace back analyzes in bioforensics and was for instance used in the 2001 Amerithrax event (Keim et al., 2004). PHRANA can be amended with whole genome sequencing data for elucidating close relationships of strains responsible for injectional anthrax among heroin consumers injecting the anthrax-contaminated drug (Keim et al., 2015).

The recent study on *B. anthracis* microevolution at aged anthrax foci (Pollino National Park, Italy) (Braun et al., 2015) amended PHRANA with whole genome sequencing in order to increase resolution power of genetic analysis (Fig. 2). For this, the genetic diversity of randomly picked *B. anthracis*-isolates was compared by 31-Loci MLVA, 4-loci SNR analysis and whole genome sequencing. MLVA-31 analysis of 114 isolates only revealed three differences, none of which in near-surface isolates. Similarly, SNR 4-loci-screening of 174 isolates yielded 18 differences with very similar percentage of occurrences of SNR-variants in near-surface (9.9%) and near-carcass (10.9%) isolates.

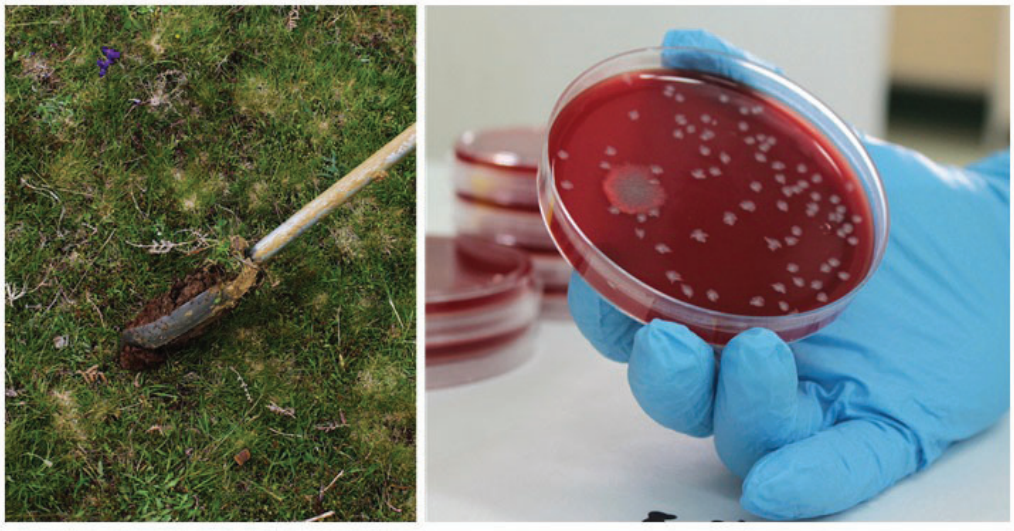

Figure 2. Application of molecular bioforensic tools used for investigating the genetic diversity of B. anthracis

Finally, genome sequencing of nine 5 cm- and 100 cm-isolates, respectively, revealed five isolate-specific SNPs, four of which only found in different isolates from 5 cm. Notably, one of these randomly-picked surface-isolates harbored two different SNPs and another one lacked plasmid pXO1. Possibly, the observed loss of pXO1 could have been an artifact of laboratory growth, however, such a loss in the laboratory was determined to be rare (Okinaka et al., 1999), and since DNA of the bacteria analyzed in (Braun et al., 2015) was extracted after a single passage, this idea can likely be discarded. While isolation of *B. anthracis* lacking pXO2 or both virulence plasmids from contaminated soils over the years is well known, natural pXO1-negative isolates are

rather rare but emerged exclusively from soil (Okinaka et al., 1999). Such strains represent an evolutionary dead end as they are likely no longer able to successfully infect new hosts due to the lack of anthrax toxins. Since pXO1-negative isolates were neither detected in infected animals during the outbreak in 2004, it is likely that this loss occurred in the soil environment. The most obvious assumption concerning the absence of pXO1 is that it reflects a soil-borne life cycle in which the plasmid was spontaneously lost during replication.

The genotyping approach called PHRANA (Progressive Hierarchical Resolving Assays using Nucleic Acids) is commonly used for outbreak and epidemiological investigations or for bioforensics of *B. anthracis* : The method comprises the consecutive application of canSNP-, MLVA and SNR analysis (Keim et al., 2004). Recently, the PHRANA fingerprinting system has been amended by whole genome sequencing (WGS) and multiple-isolate SNP comparison (Ågren et al., 2014; Keim et al., 2015; Braun et al., 2015). The left part of panel depicts simplified *B. anthracis* cells growing in chains. Plasmids pXO1 and pXO2 (open circles) and chromosome (twisted circle) are indicated with arbitrary canSNP positions (red dots), VNTRs (green bars) as well as SNRs (blue bars) and WGS SNPs (purple dots). Discriminatory power increases in every step from canSNPs to WGS SNPs and genetic resolution is dependent on the markers' intrinsic mutations rates.

The power of bioforensic tools for investigating genetic diversity in pathogens. Because of high homoplasy in MLVA and SNR markers (Keim et al., 2004), a phylogenetic explanation on the origin of length variations can hardly be given and therefore the different alleles might have originated during the course of infection as well. This would agree with the findings of Stratilo and Bader (2012), who compared SNR profiles of *B. anthracis* soil isolates distributed around carcass sites of bisons diseased from anthrax. The authors found similar distributions of SNR variations within soil samples of single carcass sites (Stratilo and Bader, 2012) which were proposed to be acquired during host passaging.

Whole genome sequencing more and more becomes the tool of choice in molecular epidemiology of infectious diseases and was already used for investigation of anthrax outbreaks (Braun et al., 2015; Keim et al., 2015; Ågren et al., 2014). Similar to variations obtained by MLVA and SNR, the SNPs might be unintentionally introduced during laboratory growth after sampling. However, the *in vitro* mutation rate of *B. anthracis* single nucleotide variations, which is due to DNA-polymerase errors, was observed to be 8.3×10^{-10} mutations/bp/generation, about 10–20 times lower than the estimated rate for *in vivo* mutations (Ågren et al., 2014). The authors did not find any mutation in three different colonies after five passages. Therefore, the possibility of *in vitro* mutation being the source of the SNPs should be negligible. Instead, given the higher mutation rate *in vivo* than *in vitro,* SNPs might be incorporated during host passaging. Regarding the whole population in a respective burial site, the occurrence of a unique SNP in any specific isolate is very rare because it must have originated from an error during the last rounds of DNA-replication before sporulation in one or only a few bacteria of the population within the dying animal. Otherwise, this SNP would be more common. It is noteworthy, that Ågren et al. (2014) found specific SNVs (Single Nucleotide Variants) for every isolate even between those from a single host animal or soil samples taken from nearby. Notably, in this Swedish outbreak, animals were treated with the antibiotic penicillin, whereas this had not been the case at Pollino National Park. The phenomenon of a higher mutation rate compared to the Pollino National Park sites can thus most easily be explained by the selective pressure applied during antibiotic treatment of the infected Swedish bovines (Ågren et al., 2014).

Conclusions. Recent experimental data support the idea that a soil-borne life cycle of *B. anthracis* exists at anthrax burial sites, which leads to microevolution increasing the genetic diversity from the depth of the carcass to the near-surface. It can be expected that improvements in technology will make possible to provide a clear-cut higher answer to this question if genotype variations are more likely due to mutation events during host infection or are a result of a soil-borne life cycle of *B. anthracis*. Currently, a model on the events at aging anthrax foci is favored which states physical diffusion to be the cause of local accumulations of endospores at or near the surface where only sporadic soil replication takes place under favorable conditions, possibly within the rhizosphere or in soil-dwelling protists as proposed earlier (Saile and Koehler, 2006; Dey, Hoffman, and Glomski, 2012).

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News

ABOUT THE INTERNATIONAL SCIENTIFIC CONFERENCE 'PROBLEMS OF EMERGENT ANIMAL DISEASES: MOLECULAR EPIZOOTIOLOGY, RAPID DIAGNOSIS AND BIOSAFETY', DEDICATED TO THE 150TH ANNIVERSARY OF THE BIRTH OF THE FIRST DIRECTOR OF THE NSC 'IECVM' PROFESSOR OLEKSANDR DEDYULIN (ODESSA, 6-10TH JUNE, 2016)

International scientific conference 'Problems of emergent animal diseases: molecular epizootiology, rapid diagnosis and biosafety', dedicated to the 150th anniversary of the birth of the first director of the NSC 'IECVM' professor Oleksandr Dedyulin organized by the National Science Centre 'Institute of Experimental and Clinical Veterinary Medicine' was held from 6 to 10 June 2016 in Odessa.

153 scientists and experts from Ukraine, the USA, Germany, Poland, Belarus, Georgia attended the forum. Among them were the representatives of agricultural and medical scientific institutions, higher education institutions, diagnostic laboratories of veterinary medicine, livestock farms and manufacturers of veterinary drugs and laboratory equipment and materials.

The main directions of the conference: issues of implementing the concept of the WHO, OIE and FAO 'One Health' in the system control emergent and economically important infectious and parasitic diseases; strategy against biological threats, the problems of biological safety and protection in the veterinary and humane medicine, biotechnology and animal husbandry; molecular epizootiology emergent infections and zoonoses, development and implementation of their monitoring, diagnosis and prognosis based on molecular genetic techniques and rapid tests; recombinant DNA biotechnology and nanobiotechnology; relevant aspects of harmonization and improvement of the regulatory framework for monitoring emergent and economically important animal diseases, as veterinary immunological products.

The vice president of Academy of Agricultural Sciences, Academician of NAAS, Baschenko M. I. opened the plenary session with greetings to the participants of the conference. Program research talk on 'Food safety and biological concepts in the context of the OIE, WHO and FAO' One Health' has been presented by made of the NSC 'IECVM' Academician NAAS Stegniy B. T.

Deputy Academician-Secretary of veterinary NAAS Corresponding Member of NAAS M.S. Mandyhra, deputy director of the State Research Institute of Laboratory Diagnostics and Veterinary Expertise candidate veterinary sciences O. M. Nevolko, representatives of scientific institutions and NAAS Ministry of Health of Ukraine, as well as higher education establishments, made in-depth presentations. All plenary and breakout sessions contained 49 presentations.

At the conference there was an exhibition of animal diseases control means, diagnostic equipment and materials. Materials of the conference were published in interagency scientific collection 'Veterinary Medicine' (№ 102) and Journal for Veterinary Medicine, Biotechnology and Biosafety (Vol. 2, №1).

Preparation and holding of the conference was supported of the Defence Threat Reduction Agency of the United States of America.

Given the performances of the participants and to improve the efficiency of research, practical implementation of scientific achievements in veterinary medicine conference took appropriate decision, shared among participants.

Contents

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Part 2. Biosafety