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A STUDY OF CASEOUS LYMPHADENITIS IN GOATS FROM A SMALLHOLDER FARM IN UKRAINE

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Summary. Caseous lymphadenitis (CLA) is a chronic infectious disease of a wide range of animal species around the world, which leads to abscesses in lymph nodes and internal organs. The aim of these studies was to characterize the *Corynebacterium pseudotuberculosis* isolate obtained from infected goat from a smallholder farm in Ukraine. Clinical observation revealed two goats with concurrent external abscesses, fever, anorexia, and weight loss. Blood samples from the suspect animals were tested by ELISA. According to the obtained results, four (18.2%) from 22 sera samples were positive and one sample was questionable. *C. pseudotuberculosis* was isolated from the lymph nodes, liver, and lungs whereas the spleen, kidney, uterus, and udder did not show any growth. Finally, the obtained isolate was characterized both by biochemical tests and using multiplex PCR based on the detection of 16s rRNA, *rpo*B, and *pld* genes of *C. pseudotuberculosis*. Disease control should include elimination of infected and suspected animals from the herd, carrying out a comprehensive disinfection, and providing constant monitoring of the entire herd. Due to the absence of available data concerning the prevalence of CLA in Ukraine, further surveillance is required and an effective disease control strategy and eradication measures need to be developed

Keywords: *Corynebacterium pseudotuberculosis*, ELISA, PCR

Introduction. *Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis (CLA) — a chronic infectious disease mainly in small ruminants and rarely in other mammalian species worldwide causing significant economic losses. The pathogen is characterized as a facultative anaerobic and facultative intracellular, Gram-positive coccobacillus bacterium, non-sporulating, non-encapsulated and non-motile that is capable of producing exotoxin phospholipase D (PLD), catalase, and has a mycolic acid-rich cell wall (Dorella et al., 2006). There are two biovars of the pathogen — Ovis (Nitrate non-reducing strains) and Equi (Nitrate reducing strains) (Almeida et al., 2017).

C. pseudotuberculosis can survive in the environment for several weeks which contributes to its further spread from herd to herd. The disease manifestation in goats and sheep includes mainly necrosis of the lymph glands (external form) or abscesses in internal organs (visceral form). Due to its zoonotic potential, the pathogen can cause granulomatous lymphadenitis or pneumonia in humans (Bastos et al., 2012; Peel et al., 1997).

Diagnosis of CLA includes observation of clinical signs, postmortem examination, pathogen isolation and identification, serology, and PCR (Binns, Green and Bailey, 2007; Pacheco et al. 2007).

Infected animals need to be isolated from the herd and thorough disinfection must be carried out. Chemotherapy predominantly is not practical except if the pathogen is detected early and long-term treatment is provided (Pratt et al., 2005). There are several vaccines against *C. pseudotuberculosis* based on bacterin or inactivated toxoid which reduce infection rates, but do not fully prevent the disease (De Pinho et al., 2021).

The information regarding CLA cases in Ukraine is very limited and additional investigations are required.

The aim of these studies was to characterize the *C. pseudotuberculosis* isolate obtained from infected goat from a smallholder farm in Ukraine.

Material and methods. Sera samples were obtained from 22 goats from smallholder private farm located in Kyiv Region, Ukraine. Antibodies to *C. pseudotuberculosis* were estimated using commercial ELISA ID Screen® *Corynebacterium pseudotuberculosis* Indirect (IDvet) for CLA. The cut-off values (S/P% = OD sample – OD negative control / OD positive control — OD negative control) were set at ≤ 40% negative, 40–50% questionable, and ≥ 50% positive.

Internal organs (lungs, liver, spleen, kidney, lymph nodes, uterus and udder) were selected from one dead goat for bacteriological studies. For *C. pseudotuberculosis* isolation sheep blood agar plates and CoryneBacAgar (Farmactive) with adding 10% of sterile bovine serum and 2% of potassium tellurite (K_2TeO_3) were used following incubation at 37 °C for 48 h in aerobic

conditions. Identification of the bacteria included biochemical test (urease, catalase, indole, H_2S production, and nitrate reduction, fermentation of glucose, lactose, maltose, mannose, and fructose) and multiplex PCR. The IndiSpin Pathogen Kit (Indical) was used for DNA extraction from *C. pseudotuberculosis* isolate according to the manufacturer's instruction. The multiplex PCR was provided using specific primers (Table 1) and DreamTaq Green PCR Master Mix (Thermo Scientific, USA) to detect 16s rRNA, *rpo*B, and *pld* genes of *C. pseudotuberculosis* according to the protocol as previously described Pacheco et al. (2007). The obtained products were discriminated by electrophoresis on a 1.5% agarose gel.

Table 1 — List of the primers, using for detection and identification of *C. pseudotuberculosis* isolate (Pacheco et al. 2007)

Primer name	Sequence 5'-3'	Target gene	Length, bp
16S-F	ACCGCACTTTAGTGTGTGTG	16s rRNA	816
16S-R	TCTCTACGCCGATCTTGTAT		
C2700F	CGTATGAACATCGGCCAGGT	*rpo*B	446
C3130R	TCCATTTCGCCGAAGCGCTG		
PLD-F	ATAAGCGTAAGCAGGGAGCA	*pld*	203
PLD-R	ATCAGCGGTGATTGTCTTCCAGG		

Results. CLA outbreak among goats was registered in a farm where 60 sheep, 25 goats, two cows, and two horses were kept together in one building. The goat flock consisted of animals with unknown epidemiological data. These new animals were not quarantined or tested for major infections. Clinical observation revealed two goats with concurrent external abscesses, fever, anorexia, and weight loss. The enlarged lymph nodes are characterized by a spherical shape, up to 12 cm in size, painless, and not fused to the skin. Blood samples from suspect animals were tested by ELISA. According to the obtained results, four (18.2%) from 22 sera samples were positive (S/P% = 72, 82, 123, and 196) and one sample was questionable (S/P% = 41).

Bacteriological findings showed the appearance of round, brilliant, dry colonies, surrounded by a hemolysis zone. The biochemical characteristics of the culture were as follows: Catalase-positive, Urease-positive, Nitrate reductase-negative, Indole-negative, H_2S-negative, fermentation of glucose, maltose, mannose, and fructose. Gram-staining of the cells showed gram-positive cocci-bacilli that are typical for microorganisms belonging to the family Corynebacteriaceae. *C. pseudotuberculosis* was isolated from the lymph nodes, liver, and lungs whereas the spleen, kidney, uterus, and udder did not show any growth.

Pathogen identification was provided by multiplex PCR (Fig. 1). All three fragments were successfully amplified that showed the presence of 16s rRNA, *rpo*B, and *pld* genes of *C. pseudotuberculosis*.

Discussion. CLA is a chronic infectious disease of a wide range of animal species throughout the world that leads to lymph node enlargement and the occurrence of abscesses in internal organs (Dorella et al., 2006). We report here a single case of CLA among goats on a farm in Kyiv Region, Ukraine.

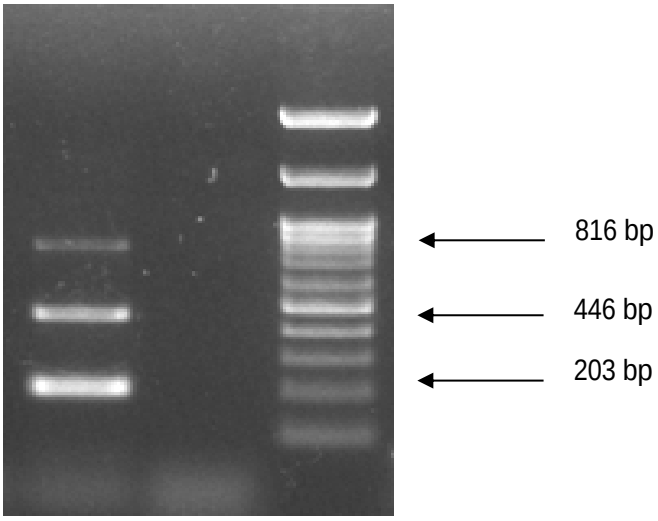

Figure 1. The multiplex PCR result of the obtained *C. pseudotuberculosis* isolate for detection of 16s rRNA, *rpo*B, and *pld* genes: S — *C. pseudotuberculosis* DNA, N — negative control (distilled water), M — DNA marker (Thermo Scientific, USA).

The animals were kept for milk production, which poses a risk to humans due to the zoonotic potential of the pathogen. In addition, infected animals were housed with other species that could also be infected with *C. pseudotuberculosis*: cattle (Mathewos and Fesseha, 2022), horses (Spier and Whitcomb, 2014; Corbeil, Morrissey and Léguillette, 2016), and sheep (Ruiz et al., 2020). However, *C. pseudotuberculosis* infection was only confirmed in goats on this farm by serology, bacteriology and molecular identification. The seroprevalence among goats on the farm was 18.2%, which was consistent with other studies (Washburn et al., 2013; Jung, et al., 2015; Costa et al., 2020). Thus, *C. pseudotuberculosis* was recovered from the lungs, liver and lymph nodes, which agreed with the results published by other authors (Baird

and Fontaine, 2007; El Damaty et al., 2023). Finally, the isolate obtained was characterized both by biochemical tests and by PCR. Disease control should include elimination of infected and suspect animals from the herd, comprehensive disinfection, and continuous monitoring of the entire herd. A control program must be established to prevent the spread of *C. pseudotuberculosis* among small ruminants in Ukraine.

Conclusions. An outbreak of CLA in the goat herd was confirmed by serological, microbiological and molecular techniques. Due to the lack of available data on the prevalence of CLA in Ukraine, further surveillance is needed and an effective disease control strategy and eradication measures need to be developed.

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ASSESSING THE EFFICACY OF ANTIPARASITIC SPRAYS

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Summary. For the control of animal ectoparasites, a large number of prophylactic and therapeutic agents with different active ingredients and routes of administration have been proposed. The persistence of ectoparasitic infections in domestic animals has led to the search for more effective drugs and innovative forms of their production. The aim of the study was to establish and experimentally confirm the efficacy of innovative ectoparasiticidal sprays for dogs and cats for prophylactic and therapeutic purposes in parasitic infections. The antiparasitic agents used in the experiments were 'PROFILINE Spray', 'INSECTOSTOP Spray', and 'Barrier-Super Insecticidal Spray'. In accordance with the tasks set, the research was carried out using visual and microscopic methods in accordance with existing practical guidelines and current methodological recommendations. In the study of ectoparasite collections, ixodid ticks (*Ixodes ricinus*), dog fleas (*Ctenocephalus canis*), and cat fleas (*Ctenocephalus felis*) were found. It was shown that the knockdown effect in all ticks began with a state of disorientation, the ticks began to move chaotically 6 hours after exposure to the experimental agents, and after 12 hours there was a complete knockdown of all experimental ixodid ticks and fleas. The high efficacy of 'PROFILINE Spray', 'INSECTOSTOP Spray', and 'Barrier-Super Insecticidal Spray' against fleas (*Ctenocephalides* spp.), lice (*Trichodectes canis, Felicola subrostratus*), lice (*Linognathus setosus*), and ixodid ticks (*Ixodes* spp.) was experimentally proven. The efficacy of the products is 100% within 60 days of application to animals. According to the results of the studies, it was found that the investigated veterinary sprays can be used for the prevention and treatment of pets with fleas, lice, and ixodid ticks

Keywords: dogs, cats, fleas, lice, ixodid ticks

Introduction. Ectoparasitic diseases of companion animals remain a pressing issue in veterinary science and practice today. These diseases represent a significant proportion of other parasitic pathologies and require a comprehensive and scientifically based solution. A relatively large number of prophylactic and therapeutic agents have been proposed to control ectoparasites, but not all of them are effective and parasites have developed resistance to some of them (Muhammad et al., 2021; Paliy et al., 2021a). This points to the need for a methodical search for more effective means and ways of using them.

Sprays are one of the modern forms of production of veterinary products, which in turn allows for more convenient use of products, their dosage, and use in limited quantities and on a limited area. At the same time, the efficacy of a veterinary product depends on the active ingredient and its quantity, as well as on excipients (Karasek et al., 2020; Paliy et al., 2021b).

Among the antiparasitic drugs used to control lice and fleas, fipronil and propoxur are the most widely used.

A new spot-on formulation containing fipronil (Eliminall®/Exproline vet™) Spot-on Solution for Dogs, Pfizer Animal Health, registered and manufactured by KRKA, d.d., Novo Mesto) was evaluated in three laboratory experiments and confirmed to be effective against fleas, ticks and lice on dogs for at least one month (Kužner et al., 2013). Effitix®, a topical ectoparasiticide containing fipronil-permethrin, provides rapid efficacy against *R. sanguineus* and *C. felis* that lasts for one month after a single application in dogs (Cvejić et al., 2017).

Frontline spray (0.25% (w/v) fipronil), Frontline spot-on for dogs (10% (w/v) fipronil) and Frontline Plus for dogs (10% (w/v) fipronil and 9% (S)-methoprene) have been confirmed in laboratory conditions to be effective against *Trichodectes canis* lice (Pollmeier et al., 2002) and spray 0.25% fipronil (Frontline Spray, Merial), 10% fipronil spot-on (Frontline Spot-on for Cats, Merial) and 10% fipronil / 12% (S)-methoprene (Frontline Plus for Cats, Merial) against *Felicola subrostratus* (Pollmeier et al., 2004).

A broad-spectrum product Effitix based on a combination of fipronil 6.1% and permethrin 54.5% has been developed that is safe and effective for dogs against ticks, fleas, and mosquitoes (Navarro et al., 2016).

Ticks have been shown to be sensitive to propoxur (Mullens et al., 2017; Thomas et al., 2018).

The aim of the study was to evaluate the efficacy of the use of antiparasitic sprays with different active ingredients for the prevention and treatment of dogs and cats.

Material and methods. The study to determine the efficacy of antiparasitic drugs on dogs and cats was conducted in the Laboratory of Veterinary Sanitation and

Parasitology of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' and at the animal shelter (Balakliya).

Modern domestic antiparasitic drugs were used in the experiments:

— 'PROFILINE Spray' (100 ml of the drug contains the active ingredient: propoxur — 0.25 g; excipients: isopropyl alcohol, polyethylene glycol — 400);

— 'INSECTOSTOP Spray' (100 ml of the drug contains the active ingredient: fipronil — 0.3 g; excipients: isopropyl alcohol, polyethylene glycol — 400);

— 'Barrier-Super Insecticidal Spray' (1 ml of the product contains the active ingredient: fipronil — 80 mg; excipients: propylene glycol, isopropyl alcohol).

Scheme of the research:

— Clinical examination of the animals in the shelter, preliminary diagnosis, collection of ectoparasites and skin scrapings for laboratory examination, constant clinical monitoring of the physiological state of the experimental animals;

— Microscopic examination of samples for the detection of parasitic pathogens in biological material, their identification and determination of the prevalence of infection in dogs and cats;

— Formation of experimental groups of animals;

— External application of drugs, individually, directly on the skin, keeping the animals in the shelter, taking samples of scrapings for laboratory testing 5, 10, 30, and 45 days after the last application of drugs. Determination of the efficacy of the drug;

— Daily clinical examination of the health of the animals throughout the experiment.

The study included 18 outbred dogs of different age groups, weighing 3 to 20 kg, and 22 outbred cats of different age groups. The animals were kept in standardized cages at an air temperature of 24.0 ± 1.5 °C, relative humidity of 40–70%, and air movement of 0.2–0.5 m/s. The animals were fed according to the diet approved by the shelter.

In accordance with the objectives, the experiments were performed by visual and microscopic methods following practical guidelines (Yiskiv, 1998; Halat et al., 2009).

The intravital diagnosis of ectoparasitoses was performed and the number of ectoparasites was determined. Identification of ectoparasitic pathogens was carried out by microscopic method. The mean intensity of the infection was determined by counting ectoparasites per 10 cm^2 of animal skin area.

Sick animals were divided into groups that were separately treated with 'PROFILINE spray' (dogs, n = 6; cats, n = 8), 'INSECTOSTOP spray' (dogs, n = 6; cats, n = 7), and 'Barrier-Super Insecticidal Spray' (dogs, n = 6; cats, n = 7).

On 5th, 10th, 20th and, 30th days after treatment, the results of the study were recorded based on examinations of treated animals, counting live ectoparasites on them, and prevalence of infection after treatment and the efficacy of the drug were determined.

Animals were clinically examined before, during, and after treatment. External examination included assessment of general appearance, skin and coat condition, measurement of body temperature, pulse and respiratory rate, examination of the eyes, mouth and ear cavities, palpation of the skin and peripheral lymph nodes.

To collect ectoparasites from the skin, the animals were restrained in a supine position. The examination of the animals' skin started with the head. Then the neck, back, sides, abdomen and limbs were examined. The wool was parted and combed during the examination. Animals were examined first with the naked eye and then with a magnifying glass. Detected ectoparasites were removed from the skin of the animals with tweezers or a rubber gloved hand. The removed ectoparasites were placed in glass dishes filled with Barbagallo's fluid (3% aqueous formalin solution in saline) or 70% ethanol. Some of the ectoparasites were delivered alive to the laboratory in tubes or containers with wet filter paper inside. The filter paper strips were moistened with boiled wateR. The tubes and containers were covered with a layer of cloth and tied. A label was placed on each tube and container.

To test dust and litter from the facilities where the treated animals were kept, a 30 g sample was placed in a cylinder or conical flask, filled with water, and mixed thoroughly. Particles floating to the surface were removed and the water was carefully poured off, leaving a precipitate. The precipitate was mixed with 20 parts of saturated sodium chloride solution and allowed to stand for 20 minutes. Then, a sample (three drops) was taken from the surface of the flotation film with a wire loop, placed on a microscope slide, covered with a coverslip, and examined under a microscope.

Prevalence (P) was defined as the ratio of the number of infected animals to the number of examined animals, expressed as a percentage:

$$P = \frac{X}{Y} \times 100$$

where: X — number of animals with detected ectoparasites, microfilariae;
Y — total number of animals.

Mean intensity (MI) of infection was determined by the number of ectoparasites per 10 cm^2 of animal skin area.

Efficacy (E) of the drug was calculated by the number of treated animals in percentage that were completely free of parasites.

In order to determine the acaricidal effect of the drugs by topical application, experimental groups of

ectoparasites were formed. They were treated separately with the test agents. The experiments were carried out in 250 cm beakers. The beakers were covered with gauze and sealed with rubber rings. The beakers were left in the room at a temperature of 20–25 °C.

The 'knockdown effect' in each beaker was determined after 6, 12, 24 hours (WHO, Geneva, 1974).

Experiments on animals were conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and in accordance with Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). The research program was reviewed and approved by the Bioethics Commission of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' in the current order.

Results and discussions. Ectoparasite collections included ixodid ticks (*Ixodes ricinus*), dog fleas (*Ctenocephalides canis*), and cat fleas (*Ctenocephalus felis*). The mean intensity (MI) of *Ixodes* ticks was 1–3 individuals per 10 cm^2 of skin area in dogs and 1–2 per 10 cm^2 of skin area in cats, and the prevalence (P) was 100%. The P of fleas in dogs was 100%, in cats — up to 100%, MI in dogs and cats — 7–10 fleas per 10 cm^2 of skin area, MI in dogs and cats — 1–2 lice per 10 cm^2 of skin area, MI in dogs — 1–2 lice per 10 cm^2 of skin area.

The onset of the 'knockdown effect' state was determined in the laboratory on ectoparasites collected from animals. The results of the determination of the rate of the onset of the 'knockdown effect' of ixodid ticks and fleas under the influence of the experimental agents are given in Table 1.

Table 1 — Efficacy of the drugs on ectoparasites when applied topically

Parasite species	Number of parasites	'Knockdown effect', after / hours		
		6	12	24
PROFILINE spray				
Ixodes ricinus	10	5	5	–
Ctenocephalus felis	10	8	2	–
Ctenocephalides canis	10	7	3	–
INSECTOSTOP spray				
Ixodes ricinus	10	5	5	–
Ctenocephalus felis	10	7	3	–
Ctenocephalides canis	10	7	3	–
Barrier-Super Insecticidal Spray				
Ixodes ricinus	10	5	5	–
Ctenocephalus felis	10	7	3	–
Ctenocephalides canis	10	6	4	–

The results presented in Table 1 show that the 'knockdown' effect in all ticks began with a state of disorientation, ticks began to move chaotically 6 hours after the effect of the products, and after 12 hours there was a complete 'knockdown' of all experimental Ixodid ticks and fleas.

The results of the determination of the therapeutic efficacy of the products studied are presented in Table 2.

Table 2 — Study of therapeutic efficacy of antiparasitic drugs

Drug	Parasite species	Infected		
		Before treatment		After treatment
		P,%	MI, insects/ 10 cm^2	Efficacy of the drug after 10 days,%
Dogs (n = 18)				
PROFI-LINE spray	*Ctenocephalus canis* *Linognathus setotus* *Trichodectes canis* *Ixodes ricinus*	100	7–8 1–2 1–2 1–3	100 (not detected)
INSECTO-STOP spray	*Ctenocephalus canis* *Linognathus setotus* *Trichodectes canis* *Ixodes ricinus*	100	1–8	100 (not detected)
Barrier-Super Insecticidal Spray	*Ctenocephalus canis* *Linognathus setotus* *Trichodectes canis* *Ixodes ricinus*	100	1–8	100 (not detected)
Cats (n = 22)				
PROFI-LINE spray	*Ctenocephalus felis* *Felicola subrostratus* *Ixodes ricinus*	100	7–10 1–2 1–2	100 (not detected)
INSECTO-STOP spray	*Ctenocephalus felis* *Felicola subrostratus* *Ixodes ricinus*	100	1–10	100 (not detected)
Barrier-Super Insecticidal Spray	*Ctenocephalus felis* *Felicola subrostratus* *Ixodes ricinus*	100	1–10	100 (not detected)

According to the results of the application of experimental products against ectoparasites of animals, it was found that already on the 2nd day after their application dead ticks, fleas and lice were found on the treated animals. Dead ectoparasites were found on animals on the 5th day, and fleas and lice were not observed on the body of animals on the 10th day. No fleas, lice or ticks were found on the body of the animals during the 30-day observation period. No complications or changes in clinical state were observed during treatment and clinical observation of experimental and control animals after administration of the drugs.

Efficacy of PROFILINE Spray, INSECTOSTOP Spray, and Insecticidal Barrier-Super Insecticidal Spray in dogs and cats infected with ectoparasites (ixodid ticks, fleas, lice) is 100%.

The results of the examination of bedding samples from the facilities where the animals treated with the test products were kept for the presence of ectoparasite larvae, nymphs and adults are presented in Table 3.

Table 3 — Examination of animal facility litter for larvae, nymphs, and adults of ectoparasites

Number of larvae and adults of ectoparasites in the litter (100 g) in the place of animal housing	
Before treatment	after the treatment of animals with the drug on the 10th day
PROFILINE spray	
17.0 ± 1.5	not detected
INSECTOSTOP spray	
10.0 ± 1.5	not detected
Barrier-Super Insecticidal Spray	
8.5 ± 1.5	not detected

As can be seen from the materials presented in Table 3, no larvae or adults of ectoparasites were found in the litter after treatment with insecticidal sprays on 10th day. The litter in the enclosures is changed every 3 days, which prevents reinfection of the animals. There is limited knowledge on ectoparasiticide resistance in Europe, as documentation on this subject is scattered and incidental. For most ectoparasite species in European countries there is no comprehensive database that provides an overview regarding the resistance situation against commonly used ectoparasiticides and possible trends over time (EMA, 2023).

Other studies have confirmed that treatment with fipronil spot-on at the recommended commercial dose rapidly reduced existing flea, tick and lice infections in dogs. Treatment provided up to 8 weeks of flea reinfection control and up to 4 weeks of tick and lice control (Kužner et al., 2013). Topical fipronil has been shown to be effective in the treatment and control of sucking and biting lice infections in laboratory rats (Diaz, 2005).

Considering all stages of the flea life cycle, the combination product with fipronil for topical application provided a high level of overall flea control, with a therapeutic effect against adult fleas and inhibition of intermediate flea stages within 12 weeks (Young et al., 2004).

All groups of dogs receiving a single topical treatment with the combination of fipronil and permethrin had significantly ($p < 0.005$) fewer fleas than untreated controls 24 and 48 hours after treatment The reduction in *C. felis* ranged from 98.4 to 100% at all time points in all studies (Fankhauser et al., 2015).

It should be noted that the use of insecticides in sublethal doses leads to the development of resistance in parasites (McNair, 2015; Zhang et al., 2019). Therefore, it is necessary to determine the effective doses of new drugs and test them before their widespread introduction into production.

Thus, our results are consistent with those of other researchers regarding the high antiectoparasitic efficacy of products containing the active ingredients propoxur and fipronil.

Conclusions. Studies on PROFILINE Spray, INSECTOSTOP Spray, and Barrier-Super Insecticidal Spray have shown that they are well tolerated by animals and do not cause any side effects or changes in the clinical state of the animals.

Laboratory studies showed a high insecticidal efficacy of the experimental products against fleas (*C. canis, C. felis*) and ticks (*Ixodes* spp.).

Clinical studies have shown high activity of the sprays against fleas (*Ctenocephalides* spp.), lice (*Trichodectes canis, Felicola subrostratus*), lice (*Linognathus setosus*) and ixodid ticks (*Ixodes* spp). The efficacy of the products is 100% within 60 days after application to animals in case of infection (*C. canis, C. felis, Ctenocephalides* spp., *Trichodectes canis, Felicola subrostratus, Linognathus setosus*) and 45 days in case of *Ixodes* spp.

According to the results of the studies, it was found that PROFILINE Spray, INSECTOSTOP Spray, and Barrier-Super Insecticidal Spray can be used for the prevention and treatment of pets affected by fleas, lice, ticks and ixodid ticks.

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Part 2. Biotechnology

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STUDY OF MORPHOLOGICAL, KARYOLOGICAL, BIOLOGICAL CHARACTERISTICS OF VIRUS-PRODUCING CELL LINES FLK-BLV (FLK-POL, FLK-71, FLK 50/100, FLK-SBBL) DURING LONG-TERM STORAGE IN THE CRYOBANK OF THE NATIONAL COLLECTION OF THE NSC 'IECVM'

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Summary. Studies have shown that, morphologically, the virus-producing cell line (FLK 50/100) consists of epithelial-like cells with a perinuclear space around the nucleus, while the FLK-71 line is represented by fibroblast-like cells with large nuclei. The karyology of FLK-71 cell lines after long-term storage was characterized by an increase in the range of chromosome number fluctuations from 40 to 64 from the data sheet of these cells before freezing (from 42 to 56). The limits of chromosome number fluctuation in FLK 50/100 decreased and ranged from 40 to 68. The antigen-producing biological activity of the subline of the reinoculated FLK-BLV culture (FLK-Pol) was preserved from passage 5 and further up to 22 passages (passage period) during the studied storage periods, namely from 55.5 months (FLK-Pol) to 201 months of storage (FLK-SBBL) and 206.5 months (FLK 50/100) in the conditions of the NSC 'IECVM' cryobank. However, for the strain FLK 50/100, up to 10 passages after thawing are required to fully recover the titers of its biological activity

Keywords: virus antigens, mitotic activity, immunodiffusion test

Introduction. Increasing the yield of bovine leukemia virus and its antigens produced in FLK-BLV cell culture as a result of leukemia virus replication in cell culture remains an urgent task for the timely diagnosis of this disease (Stegniy M., 2018; Dyakonov, 2009; Stegniy B. et al., 2019). For the diagnosis of bovine leukemia, an immunodiffusion (ID) test is used with the use of leukemia antigen, the producer of which is continuous cell culture FLK-BLV. Bovine leukemia is a chronic malignant infectious disease in which the integration of the pathogen into the body of susceptible animals causes its lifelong persistence (Van Regenmortel et al., 2000; Mamoun et al., 1983) and causes huge economic losses. The International Office for Epizootics (OIE) considers the ID test to be the main method in bovine leukemia (BLV) prevention and eradication programs today, also because the level of precipitating antibodies to the gp51 glucoprotein in cows reaches ID-positive levels 1–3 weeks after infection. The FLK-BLV cell culture was obtained in 1976 by Van der Maaten and Miller in the USA from softened fetal sheep kidney (fetal lamb kidney) cultured in monolayer (Van Der Maaten and Miller, 1976). Then, leukocytes from animals with viral lymphosarcoma were inoculated into the FLK cell culture.

Since continuous cell lines are composed of genetically heterogeneous cells, their long-term continuous passaging is often accompanied by undirected selective processes that lead to a decrease and sometimes complete loss of the necessary biological properties of the cell population (Stegniy B. et al., 2000). In order to increase the stability of the continuous lines and select the most efficient cell subpopulations, cloning is performed (Altaner et al., 1985), but the yield of fertile clones is very low. Therefore, cryopreservation with subsequent storage in the cryobank of the Pathogen Collection of the NSC 'IECVM' is very important for preserving the original properties of FLK-BLV cell lines (Stegniy M., 2009; Stegniy B., Stegniy M. and Stetsenko, 2011).

The aim of the research was to study the morphological, karyological, biological properties and antigen-producing activity of FLK-BLV virus-producing cell culture during long-term storage in the cryobank 'Collection of Pathogens of Animal Infectious Diseases', which was classified as a national heritage object by the Cabinet of Ministers of Ukaraine.

Materials and methods. The cell culture strains FLK-BLV (FLK-Pol; FLK-71; FLK 50/100; FLK-SBBL) were the study targets. To study the morphological characteristics of the continuous cell culture lines (FLK-71) and (FLK 50/100) after long-term storage in a cryobank at minus 196 °C for 17 years 9 months (FLK-71) and 19 years 8 months (FLK 50/100), they were thawed and then grown in culture mattresses or on coverslips in penicillin vials at 37 °C for one to four days, respectively, then fixed in 70% alcohol and stained with

Karachi stain, followed by visualization with an immersion light microscope.

Strains of the continuous culture FLK-BLV (FLK-Pol; FLK 50/100; FLK-71 FLK-SBBL) after long-term storage in the cryobank of the National Collection of Pathogens of the NSC 'IECVM' were thawed using a water bath at a temperature of 35–37 °C. Then, the total concentration of cells in all quadrants of the Goryaev chamber at low magnification and the concentration of preserved cells, excluding those stained with 0.2% trypan blue, were calculated. For this purpose, up to 1 cm^3 of cell suspension was added to an equal volume of 0.2% trypan blue solution, mixed thoroughly, and the Goryaev chamber was filled. The number of cells in 1 cm^3 of the suspension was determined by the formula (1):

$$X = \frac{A \times B}{0.9} \times 1{,}000 \quad (1)$$

where X — is the number of cells in 1 cm^3 of the suspension to be examined;

A — the number of counted living cells in the Goryaev chamber;

B — the dilution factor (for example, 20);

1,000 — the number of cubic millimeters in 1 cm^3;

0.9 — the volume of the Goryaev counting chamber in cubic millimeters.

Next, the cells were resuspended in a growth medium at a sowing concentration of 1.8×10^5 cells/cm^3 and seeded into culture dishes. The growth medium consisted of equal volumes of Eagle's and 199 media with 10% bovine serum.

The morphological properties of continuous cells and the dynamics of their monolayer formation after long-term storage in liquid nitrogen were studied both by daily microscopy under an inversion microscope and by making cytological preparations on coverslips. For this purpose, after defrosting, the cells were grown in penicillin vials on coverslips at 37 °C for four days. After 24, 48, 72, 96 hours of cultivation, the grown monolayer of cells was removed, rinsed in warm saline, dried with filter paper, and fixed in an ethanol-acetic solution. Cells fixed on coverslips were stored in 70% alcohol before staining with Karachi stain. Then they were rinsed with distilled water and stained with Karachi stain for 10 min. After staining, the slides were rinsed with ammonia water, dehydrated with high-concentration ethanol, finally with high-concentration ethanol-xylene, and then with pure xylene for 3 min. After air drying, the cell preparations were covered with Canadian balsam or polystyrene, followed by examination with an immersion light microscope. Mitotic activity was determined by the number of dividing cells per 1,000 cells counted and expressed in (‰) ppm (Stegniy B. et al., 2008).

In the process of cell growth, changes in the mitotic regime (mitotic activity, percentage of pathological mitoses, etc.) (Mamaeva, 1984), cytogenetic parameters and morphological state of cells were determined. The mitotic activity was determined by the number of dividing cells in relation to the total number of cells per 1,000 counted and expressed in ppm (‰).

Pathological mitoses were taken into account simultaneously with the analysis of mitotic activity (Mamaeva, 1988). The percentage number of pathological mitoses was determined by the ratio of the detected number of mitoses, and certain forms of mitotic pathology — by the ratio of the total number of pathological mitoses, which was taken as 100%.

The contamination of continuous cell cultures with fungal and bacterial microflora was checked in accordance with DSTU 4483:2005 'Veterinary Immunobiological Preparations. Methods for Determination of Bacterial and Fungous Contamination' (DSSU, 2005). For this purpose, samples of the cell suspension were taken and 0.2 cm^3 of the test sample was added to tubes with thioglycol medium and Sabouraud. Cultures on these media were incubated in a thermostat at 37 °C, and on Sabouraud medium — at room temperature (21–22 °C) for 7 and 14 days, respectively. The cultures were visually monitored daily for the growth of bacterial or fungal microflora. The absence of growth on bacterial media indicates the possibility of further use of these cell cultures for the development of biomass necessary for conservation (Stegniy B., Belokon' and Lavrik, 2005).

The study of the preservation of FLK-BLV continuous culture strains was carried out using trypan blue after thawing the cell culture; the morphology, mitotic cycle, and dynamics of the formation of a monolayer of continuous cell culture were investigated (Stegniy M. and Stegniy B., 2019; Stegniy M., Magats and Borodai, 2019).

The absence of growth on bacterial media indicates the possibility of further use of these cell cultures to develop the biomass necessary for subsequent cryopreservation to replenish the Collection and determine the antigen-producing biological activity of FLK-BLV.

After excluding contamination, it was expected that the biomass of cells would accumulate and a part of their number would be required for plating for further storage in liquid nitrogen (Belokon' et al., 1990). Virological control for the presence of diarrhea virus was carried out with specific antibodies of immune serum in a neutralization reaction according to the generally accepted method (Dyakonov, 2009).

Next, the biomass of the FLK-BLV cells was developed by repeated successive passages in the amount from 0 to 20. The leukemia virus was purified and concentrated from the culture fluid sample on ultrafiltration modules with hollow fibers. The evaluation of bovine leukemia virus antigen was carried out by activity in the immunodiffusion (ID) test and quantitative dose yield (doses of AG from 1 dm^3 of virus-

culture fluid). For this purpose, the concentrated antigen was resuspended in a phosphate-saline physiological solution with a pH of 7.0–7.2.

The reaction was performed and the results were recorded according to the 'Leaflet-inlay to the kit of components for the serological diagnosis of bovine leukemia in the immunodiffusion reaction' (No. 3272-14-0525-04/08-1/0 of 18.03.08) in 2 replicates on Petri dishes using a rectangular stamp. To determine the final titer of the antigen, serial dilutions of the experimental series were prepared from the native titer to 1:6. Phosphate-saline physiological solution with a pH of 7.0–7.2 was used as an antigen solvent. The reaction components were added to the agar wells in a volume of 40 μL.

A positive antigen titer was considered a dilution at which a clear line of precipitation with a positive control serum of two ++ was observed in the ID test. In order to compare the activity of the obtained antigens, the final antigen yield per 1 dm^3 of culture fluid was calculated. For this purpose, the term 'working titer of antigen' was used, which was considered to be the dilution of the antigen at which a positive reaction with positive serum was at ++++.

The formula (2) was used to calculate the final antigen yield per 1 dm^3 of culture fluid (X):

$$X = \frac{V_1 \times [A] \times T}{V_2} \times 100 \qquad (2)$$

where X — the amount of antigen obtained from 1 dm^3 of culture fluid, thousand doses;

V_1 — the volume of antigen actually obtained, cm^3;

[A] — the degree of concentration of the obtained antigen;

T — working titer of the obtained antigen;

V_2 — the volume of culture fluid used for antigen production

100 — coefficient for conversion to thousands of doses.

Statistical processing of the research results, mean values (M), standard deviation of the mean (m) and degree of reliability (p) was performed using the Student-Fisher method.

Results and discussions. The study of the morphological characteristics of the continuous cell culture line (FLK-71) on the third and fifth passages after thawingshowed that on the first day of cultivation, 30 to 35% of the monolayer was formed by fibroblast-like cells with large nuclei. In addition, several morphological types of cells were observed: spindle-shaped and polygonal. The morphology of the line (FLK 50/100) was represented by epithelial-like cells with a perinuclear space around the nucleus. On the first day of growth (FLK 50/100), 60% of the monolayer was formed (Fig. 1).

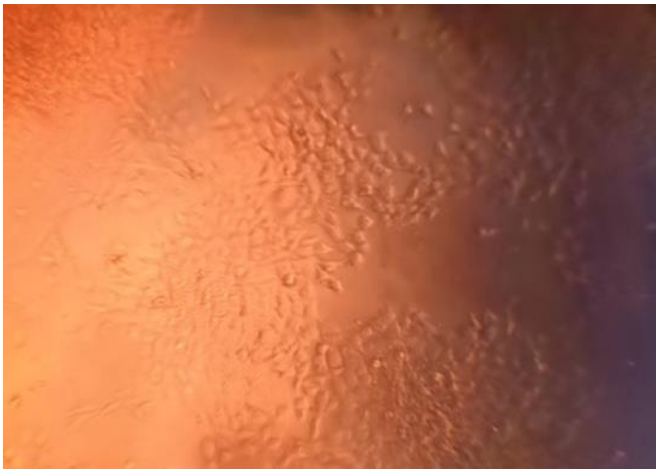

Figure 1. FLK 50/100 cell culture morphology at the third passage after thawing (magnification × 60).

In addition, 20% of the cells were at different stages of cell division, for example, metaphase plates, anaphase and telophase were observed.

After 48 hours of growth, the cells (FLK-71) formed 49% of the monolayer, and the cell cytoplasm was finely meshed. Cells in anaphase and telophase of division, as well as single multinucleated cells were observed.

The line (FLK 50/100) after 48 hours of growth formed 98% of the monolayer, which consisted of rounded cells with fine-grained cytoplasm, which occupied 35% of the cell volume, 65% — the nucleus. After 72 hours of growth, (FLK-71) cells formed 90% of the monolayer, and (FLK 50/100) cells formed 100%. The analysis of the total mitotic activity of the FLK 50/100 cell culture on the 3rd and 5th passages of cultivation showed that it was higher in the first two days of cultivation than in FLK-71 with a gradual decrease on the 3rd–4th day (Tabs 1, 2).

Table 1 — Total mitotic activity of FLK 50/100 cell culture on the 3rd passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
30.00 ± 0.82	4.95 ± 0.87	41.50 ± 0.65	6.58 ± 1.08	31.25 ± 0.75	3.96 ± 0.70	19.00 ± 0.71	3.97 ± 2.63

Table 2 — Total mitotic activity of FLK-71 cell culture on the 3rd passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
23.75 ± 1.03	2.23 ± 1.29	37.75 ± 0.48	5.28 ± 1.04	43.50 ± 1.44	4.56 ± 0.84	30.75 ± 0.95	4.96 ± 1.07

The total mitotic activity of the FLK-71 cell culture on the 3rd and 5th passages after thawing was lower than that of FLK 50/100 in the first two days of cultivation with a maximum on the third day (Tabs 3, 4).

The beginning of the monolayer destruction 30–35% was observed after 96 hours of cell growth (FLK-71), while the monolayer destruction was not observed in the (FLK 50/100) line, only an increase in the granularity of the cell cytoplasm was observed.

Table 3 — Total mitotic activity of FLK-71 cell culture on the 5th passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
26.25 ± 0.63	2.86 ± 0.96	39.25 ± 0.48	3.82 ± 0.72	44.75 ± 0.85	4.45 ± 0.85	24.75 ± 1.25	3.18 ± 1.07

Table 4 — Total mitotic activity of FLK 50/100 cell culture on the 5th passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
31.00 ± 0.82	4.01 ± 0.87	43.50 ± 1.08	8.58 ± 0.88	30.25 ± 0.75	5.96 ± 0.90	22.00 ± 0.71	5.97 ± 2.33

In addition, it should be noted that the level of mitotic activity (FLK-71) on the fifth passage after thawing was reliably 2‰ higher in the first two days of cultivation than its level on the third passage. Whereas the level of mitotic activity (FLK 50/100) on the third passage of cultivation did not differ reliably from the level of the fifth passage of this cell culture in the first three days of cultivation.

The karyologic characteristics of FLK-71 cell lines after long-term storage were characterized by an increase in the range of chromosome number fluctuations from 40 to 64 on the third passage of cultivation with a modal class of 56, and according to the passport of these cells before freezing (from 42 to 56) and 54, respectively. In the line (FLK 50/100), on the third passage of cultivation after thawing, the range of chromosome number fluctuations decreased and ranged from 40 to 68 with a modal class of 56, indicating the stabilization of culture cells (Fig. 2).

The karyological analysis of the virus-producing cell culture (FLK-Pol) on the first, second and fifth passages after thawing (from 7 months to 6 years and 7 years 2 months) of storage in the cryobank of the National Collection of Cell Cultures showed the stability of the modal class of chromosomes, which was equal to 54 from the first to the fifth passage of cultivation.

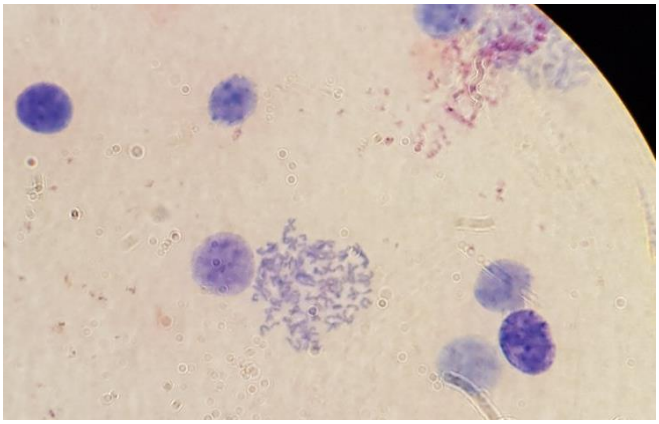

Figure 2. FLK 50/100 chromosomes on the third passage of cultivation after thawing.

The range of fluctuations in the number of chromosomes of the continuous virus-producing cell culture (FLK-Pol) on the first passage of cultivation after thawing and long-term storage in a cryobank at a temperature minus 196 °C was from 38 to 60; on the second passage — from 40 to 62; on the fifth passage — from 40 to 62.

It was proved that the addition of dimethyl sulfoxide to the culture medium accelerated the monolayer formation: on the first day of cultivation, the monolayer

was 95–100% complete, unlike the control variant, where the formation of a complete monolayer was observed only on the second day. It should be noted that in the control, monolayer destruction occurred 2–3 days earlier than in the dimethyl sulfoxide variants. All experimental groups (except for the variant with the addition of 3.0% dimethyl sulfoxide) maintained the integrity of the monolayer on the 7th day of cultivation and had a high potential for further antigen accumulation.

The titers of antigen-producing biological activity of the FLK 50/100 subline during long-term storage for 206.5 months of storage in cryobank conditions ranged from 1:1.5 to 1:4. Working titers (++++) of strain FLK 50/100 were at the same level (1:1.5 to 1:4) from 11 to 20 passages of cultivation after thawing (Table 5).

Studies have shown that the duration of storage under liquid nitrogen conditions (minus 196 °C) of a chronically infected strain (FLK-SBBL) from 4 months to 10 years did not affect the production of bovine leukemia virus after thawing and cultivation, leukemia antigen titers, its yield and volume from the first to the fifteenth passage of cultivation.

Table 5 — Activity of BLV antigens (ID) during the cultivation of FLK-BLV strains after thawing at long-term storage

Sublines FLK-BLV	Antigen titer in the immunodiffusion (ID) test	Working titer	The volume of antigen actually obtained, cm^3	Antigen yield, thousand doses per 1 dm^3
FLK 50/100 11 to 20 pas.	1:1.5 ++++ 1:2 ++++ 1:4 ++++	1:4	400	4.0
FLK 50/100 1 to 10 pas.	1:1.5 ++++ 1:2 +++ 1:3 +++	1:1.5	300	1.50
FLK-SBBL 1to10 pas.	1:2 ++++ 1:2.5 +++ 1:3 ++	1:2	420	2.00
FLK-SBBL 1 to 20 pas.	1:1.5 ++++ 1:2 +++	1:1.5	530	1.50
FLK-Pol 1 to 10 pas.	1:1.5 ++++ 1:2 ++++ 1:3 +++	1:2	385	2.00
FLK-Pol 11 to 22 pas.	1:1.5 ++++ 1:2 +++ 1:3 +++	1:1.5	440	1.50
FLK-Pol 5 to 17 pas.	1:2 ++++ 1:3 ++++ 1:4 +++ 1:6 ++	1:3	500	3.00
FLK-71 29.03.2002 11.01.2020 213	1–9	1:1.5	850	1.50
FLK-71 29.03.2002 28.01.2021	1–22	1:2	450	2.00

The antigen-producing biological activity of virus-producing strains of the FLK-SBBL cell culture after long-term storage for 201 months in the cryobank of the National Collection of Pathogens of the NSC 'IECVM' was 1:3 (++), and the working antigen titer was 1:1.5 (++++) (Table 5).

The titer of biological activity of leukemia antigen in the immunodiffusion (ID) test of FLK 50/100 strain from the 1st to the 10th passages of cultivation after thawing was 1:3 (+++), while the working titer was 1:1.5 (++++). This fact indicates that for the FLK 50/100 strain, up to 10 passages after thawing are required to fully restore the titers of biological activity.

The study of the antigen-producing biological activity of FLK-BLV strains during long-term storage in the cryobank of the National Collection of Pathogens of the NSC 'IECVM' showed that the working titers of FLK-Pol strain after 55.5 months of storage in liquid nitrogen from the 5th to the 17th passage after thawing ranged from 1:1.5 to 1:3 (++++).

The studies revealed that the preservation of biological activity titers in ID test FLK-Pol after 55.5 months of storage in liquid nitrogen ranged from 1:1.5 to 1:6 (Figs 3–5), and FLK-SBBL from the 1st to the 10th passages after 81.5 months of storage was 1:2, after 129.5 months of storage in liquid nitrogen — 1:1.5.

Figure 3. Lines of precipitation in ID test of the strain FLK-Pol after 55.5 months of storage in cryobank conditions from passage 5 to 17 after thawing. Dilutions from 1:1.5 to 1:3 (++++).

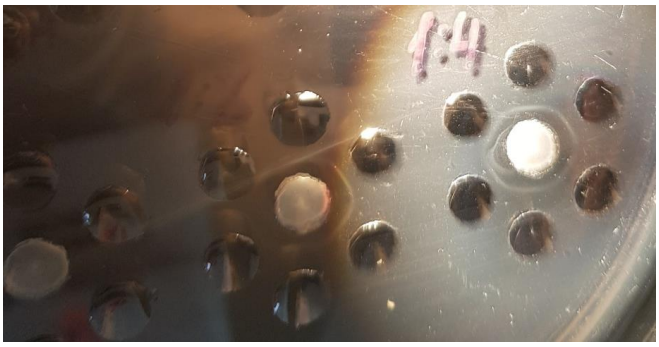

Figure 4. Lines of precipitation in ID test of the strain FLK-Pol after 55.5 months of storage in cryobank conditions from 5 to 17 passages after thawing. Dilution 1:4 (+++).

Figure 5. Lines of precipitation in ID test strain FLK-Pol after 55.5 months of storage in cryobank conditions from passage 5 to 17 after thawing. Dilution 1:6 (++).

Thus, the antigen-producing biological activity of the FLK-BLV strain (FLK-Pol) was preserved from the 5th passage and further up to 22 passages (passage period) during the studied storage periods, namely from 55.5 months (FLK-Pol) to 201 months of storage (FLK-SBBL) and 206.5 months (FLK 50/100) in the conditions of the NSC 'IECVM' cryobank. However, for the FLK 50/100 strain, up to 10 passages after thawing are required to fully restore the titers of its biological activity.

Conclusions. 1. It has been demonstrated that the virus-producing cell line (FLK 50/100) morphologically consists of epithelial-like cells with a perinuclear space around the nucleus, whereas the FLK-71 line is represented by fibroblast-like cells with large nuclei.

2. The karyology of FLK-71 cell lines after long-term storage was characterized by an increase in the range of chromosome number fluctuations from 40 to 64 compared to the passport data of these cells before freezing (from 42 to 56). The range of chromosome number fluctuations in FLK 50/100 decreased and ranged from 40 to 68.

3. The karyological analysis of the virus-producing cell culture (FLK-Pol) on the first, second and fifth passages after thawing showed a stable fluctuation in the number and modal class of chromosomes, which was 54 from the first to the fifth passage of the cultivation.

4. Studies have shown that the duration of storage under liquid nitrogen conditions (minus 196 °C) of a chronically infected strain (FLK-SBBL) from 4 months to 201 months of storage did not affect the production of bovine leukosis virus after thawing and cultivation, leukemia antigen titers, its yield and volume from the first to the fifteenth passage of cultivation.

5. The antigen-producing biological activity of the continuous culture of FLK-BLV (FLK-Pol) was preserved from the 5th passage and further up to 22 passages (passage period) during the studied storage periods, namely from 55.5 months (FLK-Pol) in the conditions of the NSC 'IECVM' cryobank. The antigen-producing biological activity of the strain (FLK 50/100) was fully restored only after 10 passages of its cultivation after thawing.

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DEVELOPMENT OF IN-HOUSE DIAGNOSTIC TOOL FOR THE DETECTION OF ANTHRAX GENETIC MATERIAL IN REAL-TIME PCR

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Summary. This paper represents preliminary trials of the 'Anthrax-DNA-test', diagnostical tool for the detection of anthrax DNA. It includes recombinant positive controls *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* for the detection of anthrax plasmid markers, as well as *p-dhp61-CR2.1-TOPO*, positive control for the detection of *Bacillus anthracis* chromosomal marker. Besides, three mixtures of primers and probes for the detection of each genetic marker (*dhp61, pagA*, and *capC*) and ready-to-use 'RT-PCR MasterMix' PCR diluent were also included. Concentrations of $MgCl_2$ and Taq-polymerase obtained during qPCR validation procedure were considered when preparing the diluent. To determine specificity, qPCR was conducted with heterological panel of DNA of pathogenic bacteria and viruses causing diseases with similar to anthrax clinical signs. To determine repeatability of the results when using 'Anthrax-DNA-test' PCR test kit, samples were studied twice. The sensibility of the kit was analyzed by serial dilutions of *p-dhp61-CR2.1-TOPO*, *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* plasmid DNAs containing fragments of anthrax chromosome and plasmids. To compare the tool's ability to identify anthrax DNA, classical PCR was carried out using ANT-PA_F/R and ANT-CAP_F/R primers recommended by OIE for the detection of pXO1 and pXO2 plasmid DNA. Sensitivity testing has shown that the test kit is able to identify all positive samples. It has been found that the diagnostics tool detects anthrax DNA in recombinant positive control samples containing *B. anthracis* chromosomal and plasmid DNA fragments in serial dilutions from 1:100 to 1:1,000 with Ct values of 25.29–34.70. The specificity of this diagnostic tool is proved by the absence of Ct in heterological samples. Besides, repeatability of trial results has been found, which is proved by complete congruence in duplicates with each of the tested sample

Keywords: *Bacillus anthracis*, plasmid, validation

Introduction. Anthrax is a zoonotic disease to which mainly grazing herbivores, but also omnivores, carnivores and human are susceptible. It is caused by Gram-positive, spore-forming facultative anaerobic rod *Bacillus anthracis* (Purcell, Worsham and Freidlander, 2007; Hoffmaster et al., 2002; Keim et al., 2004). Depending on the way of transmission, it can cause cutaneous, gastro-enteritic of pulmonary forms of anthrax (WHO, FAO and OIE, 2008). Under adverse conditions the causative pathogen *B. anthracis* is able to form spores that may remain viable in the environment, especially in soil, for many decades (Martin, Christopher and Eitzen, 2007). When spores penetrate to host organism, they turn to vegetative form, reproduce and therefore cause the disease. The ability to produce toxins and form capsule in hosts organism, which protects bacterial cell from phagocytosis, are key virulence factors of *B. anthracis.* Genes responsible for capsule formation are located on pXO2 plasmid, while pXO1 plasmid genes encode synthesis of toxins. Both these plasmids together with chromosome form anthrax genome (Mock and Fouet, 2001).

Together with classical bacteriological and serological methods, classical polymerase chain reaction and real-time PCR (quantitative PCR, qPCR) are commonly used for express diagnostics of anthrax. Usually, the diagnosis of anthrax is performed following a specific PCR test to detect gene fragments which are specific to plasmids pXO1 and pXO2, respectively (Janzen et al., 2015). However, due to the high degree of homology between *B. anthracis*, *B. cereus* and *B. thuringiensis* (Helgason et al., 2000), the detection of only plasmid markers is insufficient for the diagnosis of anthrax. It should also be noted that plasmids pXO1 and pXO2 can be lost by microorganisms or may also be present in closely related bacteria of the *B. cereus* group (Hurtle et al., 2004; Pannucci et al., 2002), which serves as an additional factor complicating the diagnosis of anthrax.

Currently, using PCR test kits allows to make diagnostic analyses in a laboratory significantly simpler and faster. However, there is no effective domestic PCR diagnostic tool for the detection of *B. anthracis* genome in Ukraine. Besides, using foreign analogs is quite expensive due to the high cost price of reagents and expenses for their transportation. Also, it is necessary to note that the transportation issue has become especially acute in Ukraine due to the lack of airborne routes and complicated logistics caused by the Russian military aggression.

Therefore, the key task of our work was to create a domestic diagnostic tool designed to detect not only plasmids pXO1 and pXO2 in the tested material but also a highly specific region of the *B. anthracis* genome present only in the chromosome of this pathogen.

Materials and methods. Testing of the diagnostic tool was conducted using previously-developed recombinant

positive controls, namely *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* in various dilutions (Fig. 1) (Biloivan et al., 2018). The positive control sample *p-dhp61-CR2.1-TOPO* for detecting of anthrax *dhp61* specific chromosomal marker, developed by Antwerpen et al. (2008) and kindly provided by colleagues from the Bundeswehr Institute of Microbiology (Munich, Germany), was also included as the component of this test kit. In this process, plasmid DNA *p-dhp61-CR2.1-TOPO*, *p-pagA-TZ57R/T*, and *p-capC-TZ57R/T* were used at various dilutions (1:100, 1:1,000, 1:10,000), as well as positive and negative control samples.

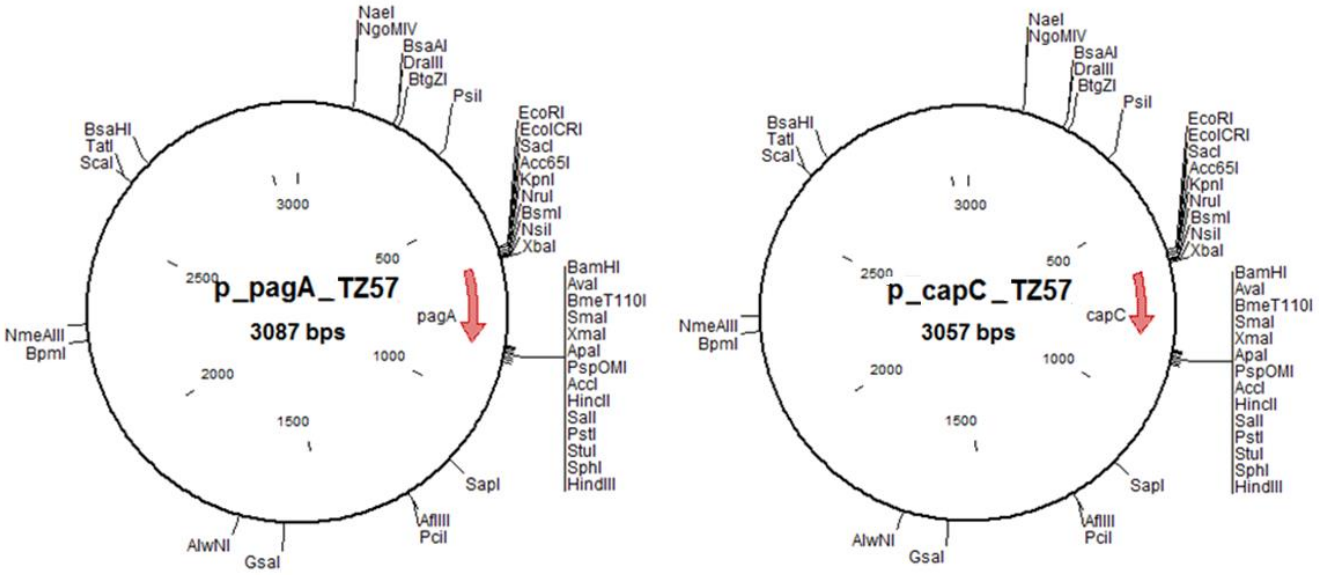

Figure 1. Plasmid maps of recombinant positive control assays *p-pagA-TZ57R/T* (left) and *p-capC-TZ57R/T* (right) for the detection of pXO1 and pXO2 anthrax plasmids, respectively.

The reaction mix was prepared using *TaqMan* reagents and concentrations of its components ($MgCl_2$ and *Taq*-polymerase) were optimized at previous validation stages of our studies (Beloyvan et al., 2019; Biloivan et al., 2019).

The working mixture for real-time PCR was prepared based on the number of tested samples plus one sample, according to the following scheme presented in Table 1.

Table 1 — The reaction mix prepared for the RT-PCR

Component	Final concentration	1× per reaction, µl
RT-PCR MasterMix	1×	18.5
The mixture of primers and probe for the detection of *dhp61, pagA* or *capC* markers	200 nM	1.5
DNA (sample or control)		5

Species specificity was identified using heterological DNA samples of various pathogens with cause infectious diseases with similar to anthrax clinical signs (Table 2).

In order to assess the reproducibility of the results obtained when using the developed test kit, the sample analysis was conducted twice.

Table 2 — Heterologous panel used to determine the species specificity of the diagnostic tool for the detection of anthrax DNA

No.	Pathogen	Strain number
1	*Acinetobacter baumanii*	B431
2	*Brucella spp*	03-0391
3	*Burkholderia cepacia*	P112
4	*Burkholderia mallei*	05-0580
5	*Burkholderia pseudomallei*	Jun 88
6	*Burkholderia thailandensis*	P412
7	*Campylobacter jejuni*	B1229
8	*Candida albicans*	B885
9	*Chlamydophila pneumoniae*	No data
10	*Citrobacter freundii*	B22
11	*Clostridium perfringens*	B888
12	*Coxiella burnetii*	Nine Mile
13	*Enterobacter aerogenes*	B16
14	*Enterococcus faecalis*	B871
15	*Escherichia coli*	B893
16	*Francisella tularensis holarctica*	F49
17	*Haemophilus influenzae*	B895
18	*Klebsiella pneumoniae*	B896
19	*Legionella pneumophila*	IMB 072813
20	*Listeria monozytogenes*	B435
21	*Moraxella catarrhalis*	B433
22	*Mycobacterium tuberculosis*	No data

Table 2 — continuation

No.	Pathogen	Strain number
23	*Neisseria meningitidis*	B1232
24	*Propionibacterium acnes*	B438
25	*Proteus mirabilis*	B23
26	*Salmonella typhi*	20-3267
27	*Serratia marcescens*	B14
28	*Shigella dysenteriae*	B476
29	*Staphylococcus aureus/SEB*	B946
30	*Staphylococcus epidermidis*	B26
31	*Stenotrophomonas maltophilia*	B918
32	*Streptococcus pneumoniae*	B847
33	*Streptococcus pyogenes*	B846
34	*Vibrio cholerae*	B962
35	*Yersinia enterocolitica*	Y105
36	*Yersinia pestis*	02. Apr
37	*Clostridium sporogenes*	DSMZ795
38	*Monkey pox virus*	MSF-6
39	*Cowpox virus*	VACV-0273/2004
40	*Chickenpox virus*	No data

The real-time amplification reaction was set up according to the protocol outlined in Table 3.

Table 3 — The real-time PCR amplification protocol for the detection of *B. anthracis* genetic material

Stage	Amplification mode	Number of cycles
Activation	95 °C — 5 min	1
Denaturation Annealing Elongation	95 °C — 15 sec 60 °C — 20 sec 72 °C — 40 sec	40
Final elongation	72 °C — 1 min	1

The specificity of the test kit was determined by comparing to primers recommended by OIE for the detection of anthrax plasmid DNA fragments: PA5/8 (*pag* gene of pXO1 plasmid) and 1234/1301 (*cap* gene of pXO2 plasmid) (WOAH, 2023a, 2023b; Hutson et al., 1993; Beyer et al., 1995; WHO, FAO and OIE, 2008).

The primer pair PA5/8 flanks a 596-base pair region of the *pag* gene of pXO1 plasmid:

PA5, 5'-TCCTAACACTAACGAAGTCG-3';

PA8, 5'-GAGGTAGAAGGATATACGGT-3'.

The primer pair 1234/1301 flanks an 846-base pair region of the *cap* gene of pXO2 plasmid:

1234, 5'-CTGAGCCATTAATCGATATG-3';

1301, 5'-TCCCACTTACGTAATCTGAG-3'.

The results of conventional PCR using PA5/8 and 1234/1301 primers were recorded by horizontal gel electrophoresis. A sample was considered positive for the presence of anthrax pXO1 and pXO2 plasmid DNA if yellow-hot 596 bp (for the *pag* gene of pXO1 plasmid) and 846 bp (for the *cap* gene of pXO2 plasmid) bands were visible on the gel and negative if they were absent, respectively.

Results. The developed test kit includes the following components:

(1) 'RT-PCR MasterMix' — 1 (2) tubes of 1 ml each;

(2) primer and probe solutions for detecting of *dhp6*, *pagA* and *capC* markers (10 pmol/μL) — 1 tube each, 0.03 (0.06) ml (each);

(3) deionized water — 1 (2) tubes of 0.5 ml each;

(4) positive control samples for detecting of *dhp61* chromosomal marker as well as *pagA* and *capC* plasmid markers (for 5 or 10 reactions) — 1 tube each, 0.1 (0.2) ml (each).

The 'RT-PCR MasterMix' solution includes $MgCl_2$, deoxyribonucleotide triphosphates (dNTPs), and Taq-polymerase. The concentrations of $MgCl_2$ and Taq-polymerase were optimized during a previous stage of our studies (Beloyvan et al., 2019; Biloivan et al., 2019).

For convenience in a diagnostic laboratory setting, primer and probe mixtures were prepared in equal volumes, with three mixtures in total for each of the genetic markers (*dhp61*, *pagA*, and *capC*). Additionally, the test kit includes positive control samples for detecting the plasmid markers of *B. anthracis* (*pagA* and *capC*), which were prepared previously (Biloivan et al., 2018).

The positive control sample *p-dhp61-CR2.1-TOPO* for detecting the chromosomal marker of the anthrax agent, developed by Antwerpen et al. (2008) and kindly provided by colleagues from the Bundeswehr Institute of Microbiology, is also included in the test kit. Each component is aliquoted into tubes in quantities sufficient for conducting 50 analyses.

It has been found that the test kit is able to detect anthrax DNA in plasmid DNA samples *p-dhp61-CR2.1-TOPO*, *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* which contain fragments of DNA markers of the chromosome and plasmids of *B. anthracis*, respectively, in dilutions ranging from 1:100 to 1:10,000 with Ct values between 25.29 and 34.70 (Table 4).

At the same time, no amplification product was observed in heterologous samples (Ct values were absent), demonstrating the specificity of this test system. Furthermore, the repeatability and reproducibility of the results have been established, as evidenced by their complete agreement in two replicates with each tested sample.

Thus, the preliminary testing of the developed 'Anthrax-DNA-test' has demonstrated that it meets the OIE requirements in terms of specificity, sensitivity, and reproducibility (WOAH, 2023a, 2023b).

Table 4 — Trial results of the 'Anthrax-DNA-test' diagnostic tool

No.	Assay	PA5/8 and 1234/1301 primer pairs	The first replicate, Ct	The second replicate, Ct
1	*p-dhp61-CR2.1-TOPO* plasmid DNA diluted 1:100	positive	28.90	29.10
2	*p-dhp61-CR2.1-TOPO* plasmid DNA diluted 1:1,000		31.30	31.40
3	*p-dhp61-CR2.1-TOPO* plasmid DNA diluted 1:10,000		34.70	34.40
4	*p-pagA-TZ57R/T* plasmid DNA diluted 1:100		27.28	27.39
5	*p-pagA-TZ57R/T* plasmid DNA diluted 1:1,000		30.73	31.46
6	*p-pagA-TZ57R/T* plasmid DNA diluted 1:1,000		33.69	33.70
7	*p-capC-TZ57R/T* plasmid DNA diluted 1:100		26.06	25.29
8	*p-capC-TZ57R/T* plasmid DNA diluted 1:1,000		29.14	28.96
9	*p-capC-TZ57R/T* plasmid DNA diluted 1:1,000		32.86	32.36
10	DNA of *Acinetobacter baumanii*	negative	Ct is absent	Ct is absent
11	DNA of *Brucella* spp.			
12	DNA of *Burkholderia cepacia*			
13	DNA of *Burkholderia mallei*			
14	DNA of *Burkholderia pseudomallei*			
15	DNA of *Burkholderia thailandensis*			
16	DNA of *Campylobacter jejuni*			
17	DNA of *Candida albicans*			
18	DNA of *Chlamydophila pneumoniae*			
19	DNA of *Citrobacter freundii*			
20	DNA of *Clostridium perfringens*			
21	DNA of *Coxiella burnetii*			
22	DNA of *Enterobacter aerogenes*			
23	DNA of *Enterococcus faecalis*			
24	DNA of *Escherichia coli*			
25	DNA of *Francisella tularensis holarctica*			
26	DNA of *Haemophilus influenzae*			
27	DNA of *Klebsiella pneumoniae*			
28	DNA of *Legionella pneumophila*			
29	DNA of *Listeria monozytogenes*			
30	DNA of *Moraxella catarrhalis*			
31	DNA of *Mycobacterium tuberculosis*			
32	DNA of *Neisseria meningitidis*			
33	DNA of *Propionibacterium acnes*			
34	DNA of *Proteus mirabilis*			
35	DNA of *Salmonella typhi*			
36	DNA of *Serratia marcescens*			
37	DNA of *Shigella dysenteriae*			
38	DNA of *Staphylococcus aureus/SEB*			
39	DNA of *Staphylococcus epidermidis*			
40	DNA of *Stenotrophomonas maltophilia*			
41	DNA of *Streptococcus pneumoniae*			
42	DNA of *Streptococcus pyogenes*			
43	DNA of *Vibrio cholerae*			
44	DNA of *Yersinia enterocolitica*			
45	DNA of *Yersinia pestis*			
46	DNA of *Clostridium sporogenes*			
47	Monkey pox virus DNA			
48	Cowpox virus DNA			
49	Chickenpox virus DNA			

Conclusions. The key objective of our work was to create a in-house diagnostic tool designed to detect the anthrax genetic material. One of the distinguishing features of the developed 'Anthrax-DNA-test' kit is its capability to detect not only the pXO1 and pXO2 plasmids but also the chromosomal marker *dhp61*, which is highly specific and present only in the *Bacillus anthracis* chromosome. This feature is of great importance when distinguishing the anthrax pathogen from other closely related bacteria. The developed test kit, in terms of specificity, sensitivity, and reproducibility, is comparable to the method recommended by the OIE and, after the completion of the registration process, will be used for the express diagnosis of anthrax in Ukraine.

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PORCINE REPRODUCTIVE AND NEONATAL INFECTIONS: IMPORTANCE AND THREATS OF BACTERIAL VIROPHORIA

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Summary. The provisions of the doctrine of transfer the epizootic process of dangerous viral infections to the enzootic process and their rooting in pig production through the integration of their pathogen into the pig microbiome in the form of comorbid viral and bacterial infections are substantiated. The aim of the study is to systematize the bacterial virophoria in the epizootology of porcine reproductive and neonatal infections (PRNI) as a component of the enzootic cycle of emergent infections in the pig industry of Ukraine. Classical swine fever (CSF) virus: attenuated strain 'IECVM-03'; Aujeszky's disease (AD) virus: epizootic strains of Ukrainian origin of AD virus '18v-UNDIEV'; Teschen disease (TD) virus: epizootic strain 'Bucha'. Epizootic strains: pasteurella bacteria, streptococcus, lacto- and bifidobacteria. According to the results of the study, it was found that the rotating magnetic field of the right direction promoted the adsorption of the CSF virus on pasteurella cells. The Aujeszky's disease virus was adsorbed on the bacteria *Salmonella choleraesuis* No. 34, *Bacterium bifidum* and *Lactobacillus casei* with an efficiency of 15–45% in the pH range of 8.5–9.5, at neutral pH (7.4) no more than 1.5% of the virus was adsorbed, and at acidic pH (3.0) the AD virus was not adsorbed et all. On bacteria *Pasteurella multocida* No. 7, AD virus was adsorbed in the pH range of 8.5–9.5 with an efficiency of no more than 1.5%; at neutral pH (7.4), up to 50% of the virus was adsorbed, and at acidic pH (3.0), no more than 1.5% of AD virus was adsorbed. The interaction of TD virus with bifidobacteria inhibited viral reproduction in the body of infected polecats, but preserved the reproductive activity of teschovirus in the presence of streptococci. The rooting of dangerous viral infections (AD and TD, circovirus and parvovirus infections, reproductive and respiratory syndrome, and endemic porcine diarrhea) in pig production has always been accompanied by the 'engraftment' of their pathogens in the microbiome of pig production facilities in the form of comorbid (i. e. clinically manifested) and/or associated infections (i. e. similar to the group of Minimal Residual Human Diseases — Maladie Résiduelle Minimale, MRD). A key role in the establishment of these diseases and the formation of their stationary centers in pig production is played by the virophoria of bacteria synergistic with their pathogens, in particular as part of the etiologic microflora of reproductive and neonatal infections in pigs

Keywords: virus adsorption, virulence, viral-bacterial infections, rooting of emergent pathogens

Introduction. Over the past decade, we have been studying the mechanisms of infectious diseases in industrial pig production associated with outbreaks of lethal fevers in rearing groups and reproductive disorders in the nucleus of the industrial pig herd. Already in the first 2–3 years of research, we became convinced that Aujeszky's disease (AD), Teschen's disease (TD), porcine circovirus (PCV) and porcine parvovirus (PPV) infections, reproductive and respiratory syndrome (PRRS), and porcine endemic diarrhea (PED), sometimes also certain viral vaccines against them, as well as classical swine fever (CSF) have been implicated in one way or another in the anamnesis of comorbid viral-bacterial pneumoenteritis in young animals and reproductive disorders in sows and boars.

Due to the similarity of epizootic manifestations and in order to standardize control protocols, we proposed to refer these diseases to porcine reproductive and neonatal infections (PRNI).

Unlike the bacterial component, the viral component of the etiology of these infections is clinically manifested almost exclusively in newborns and pigs after childbirth: although not always, and only for a short period of time — during exacerbation of PRNI. This exacerbation usually becomes emergent, as the use of antiepizootic drugs and measures periodically requires the search for unconventional approaches to antibiotic, chemotherapy, sero- or vaccine prophylaxis.

Another feature of PRNI is their 'tendency' to form stationary foci, in which periods of enzootic exacerbation are cyclically replaced by relative 'quiescence'. Today, few people pay attention to this, but in such foci PRNI manifest as comorbid infections of viral and bacterial etiology. Moreover, this manifestation depends not only on the composition of the etiologic viral-bacterial consortium of PRNI, but also on the action of a particular causative factor. In these cases, we often encountered the phenomenon of bacterial virophoria,

when certain bacterial species showed signs of adsorption of the corresponding viral agent from the consortium on their surface. Unfortunately, in our opinion, the above mentioned known data remain insufficiently systematized.

The aim of our work was to systematize bacterial virophoria in the epizootology of PRNI as a component of the enzootic cycle of rooting of pathogens of emergent infections in the pig industry of Ukraine.

Materials and methods. The mechanisms of virophoria formation were studied on the model of the oldest, according to scientific data, formerly exotic, but now familiar to the veterinary service of Ukraine viruses — the pathogens of AD and TD (Rudyk, 1995; Korolov, 2011; Derevianko, 2019).

This choice was made not only because of the indisputable long-standing rooting of these pathogens in Ukraine, but also because of the tradition of their use as surrogate models in the study of certain issues of epizootology of African swine fever (ASF) and foot-and-mouth disease (FMD), respectively (Frost et al., 2023; Harada et al., 2015).

Aujeszky's disease virus (ADV): epizootic strains of Ukrainian origin AD '18v-UNDIEV' isolated in 1968 (Poltava Region) and '1082' isolated in 2010 (Donetsk Region). These viruses, after passaging in laboratory mice, secrete hemagglutinins of mouse erythrocytes, which provides the possibility of in vivo studies in the format of a budget-saving epizootic model 'virus–mouse'. Before and during use, the viral suspensions were titrated in transplanted porcine cell cultures by the classical method (Reed and Muench, 1938) and in the haemagglutination assay (HA) and the hemagglutination inhibition assay (HIA) following Tetsu et al. (1989) * in accordance with the relevant standard operating procedure (SOP) of the NSC 'IECVM'. Viral suspensions with an activity of 6.5–7.5 lg $TCID_{50}$/ml (512–1,024 HAO_{50}/ml) were used to model virophoria and control its effect on pathogenicity of agents for mice.

Teschen's disease virus (TDV): epizootic strain 'Bucha' isolated in 2003 (Poltava Region). A trophovariant of the pathogen adapted to guinea pigs was used in a cost-effective epizootic model of peripheral neuropathogenicity of *Teschovirus* (Buzun, 2021); its infectious activity was controlled in continuous cultures of pig cells according to the relevant SOP of the NSC 'IECVM'. Viral suspensions with an activity of 7.0–7.5 lg $TCID_{50}$/ml were used in the experiments.

Classical swine fever virus (CSFV): attenuated strain 'IECVM-03' isolated in 2003 (Sumy Region) was grown and tested in the continuous culture of pig cells PK-15 according to the relevant SOP of the NSC 'IECVM'. Viral suspensions with an activity of 4.5–5.5 lg FFU_{50}/ml were used in the experiments. To control their infectious activity, as well as the activity of TD and AD pathogens (in accelerated variants), the method of fluorescent antibodies (FA) was applied, using appropriate diagnostics according to the SOP of the NSC 'IECVM'.

To model the virophoria of these pathogens, epizootic strains were used: of bacteria isolated in 2008 and 2012 from pigs — *Pasteurella multocida* No. 7 strain (2006, Poltava Region) and *Streptococcus suis* No. 53 strain (2009, Luhansk Region), *Salmonella choleraesuis* No. 34 strain (2011, Donetsk Region). Production strains of the probiotic bacteria *Bifidobacterium bifidum* and *Lactobacillus casei* ('resident microflora') were also used. The stock cultures of all bacteria were used at a concentration of 1.2×10^9 CFU/ml (based on the results of titration on dense medium).

Among the physical environmental factors that, according to the literature, can activate bacterial virorrhization (factors affecting particle agglutination) (Ortega-Vinuesa and Bastos-González, 2001), this series of studies tested (a) for the CSF virus and pasteurella, rotating magnetic fields of the right and left directions and (b) for AD and TD pathogens and pasteurella, streptococci, bifido- and lactobacteria, the acidity of the medium (pH). The generator of rotating magnetic fields was a device by Hrabina et al. (2009), and buffer solutions with appropriate pH and salt molarity served as acidity factors.

In the *in vivo* models, all manipulations with outbred laboratory mice and guinea pigs were performed in accordance with the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and basic bioethical principles (Simmonds, 2017). The experiments were conducted in compliance with the principles of humanity stated in the European Council Directive 2010/63/EU (CEC, 2010).

Research results. *A. Study of the CSF virus virophoria on pasteurella.* The potential physical impact on the interaction between the CSF virus and pasteurella was studied using right (RR) and left (LR) rotating magnetic fields. To do this, we used the device of Hrabina et al. (2009) (Fig. 1a), which generates asymmetric magnetic fields. Virus-bacterial mixtures containing the CSF virus (strain 'IECVM-03'; final concentration 4.5–5.0 lg FFU_{50}/ml †) and pasteurella *P. multocida*

* With 0.5–1.0% mouse erythrocytes, total reaction volume — 0.075 cm^3, sample dilution step — 2, solvent — phosphate-gelatin buffer solution (pH 7.4), V-well plates, incubation at room temperature, recording of results after the control erythrocytes settle into the 'button'

† lg FFU_{50}/ml — the decimal logarithm of the extreme dilution of the virus-containing suspension under study, which still contains 50% of the test slides with focus-forming units of the CSF virus

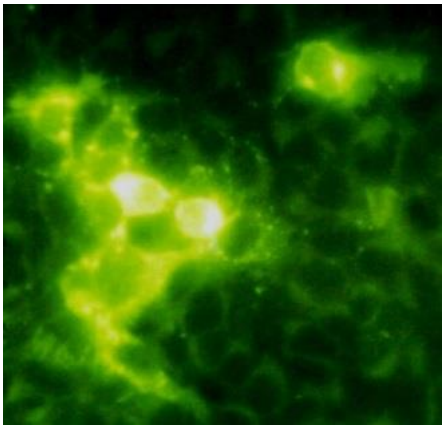
(strain '7', activity 5×10^5 BC_{50}/ml[‡]) on a buffered saline solution, each in volumes of V = 5.0 and 7.0 cm^3, were exposed to a rotating magnetic field of θ =0.2 mTl. The samples were exposed to the right (n = 17) and left (n = 11) rotating magnetic fields for 25 min. The control samples (CS) (n = 17) were identical to the experimental ones in all respects: they were exposed for 25 min with the device turned off. After exposure, all precipitates of the mixtures (pasteurella) were washed in buffered saline solution by a single centrifugation (1,000 gX, 30 min). The washed bacterial suspensions of the RR samples were divided into three parts: the first of them was controlled for the presence of the CSF virus in the bacterial precipitate in parallel with the precipitates of the LR and CS. The second and third parts of the RR samples were immediately exposed in a thermostat at 40.0 ± 0.5 °C for 3 and 5 days, respectively, after washing, and only then were they monitored for the presence of the virus. In all cases, the titration of CSF virus was performed according to the standard procedure by FA after virus isolation in the continuous PK-15 cells on slides (Fig. 1b). Two of the 17 RR-precipitates were tested by polymerase chain reaction (PCR) for CSF, using commercial primers to identify CSF virus adsorbed on pasteurella (Fig. 1c).

By both tests, CSF virus was detected on pasteurella only in the bacterial RR sediments: in 11 samples out of 15 examined by FA and in one out of two by PCR. The geometric mean titer of the virus in 9 positive RR-precipitates was 2.5 ± 0.3 $lgFFU_{50}$/ml (p < 0.01). On the 3rd and 5th day after exposure at 37 °C, RR-precipitates of virophoric bacteria contained remnants of infectiously active CSF pathogen: the geometric mean virus titers were 1.1 ± 0.8 $lgFFU_{50}$/ml (n = 4) and 1.0 ± 0.9 $lgFFU_{50}$/ml (n = 2), respectively.

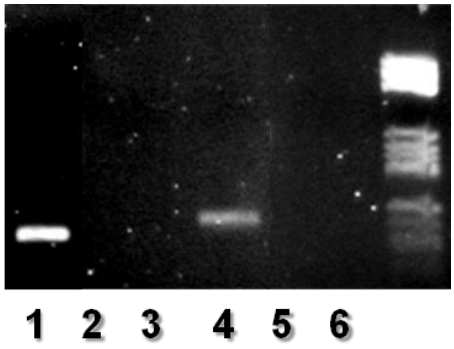
At the same time, the initial viral suspension of the pathogen at 40 °C was completely inactivated after 35 hours of exposure (n = 3, p < 0.01). All LR- (n = 11) and CS-precipitates (n = 17) were negative for CSF in FA and PCR tests. Moreover, 7 out of 11 LR sediments did not even contain live bacteria. These results to some extent coincide with the data on the phenomenon of 'Gränder's water'[§], which is manifested in bacteria-contaminated water under the influence of weak magnetic irradiation from a device patented in Austria (Liu et al., 2022).

Thus, a rotating magnetic field of the right direction with a power of θ = 0.2 mTl promotes the adsorption of the CSF virus on pasteurella cells — stimulates the virophoria of these bacteria. At the same time, the virus adsorbed on bacteria acquires significant resistance at temperatures up to 40 °C inclusively.

a



b



c

Figure 1. Summary data on the use of a right-rotating magnetic field for modeling the virulence of the CSF pathogen on pasteurized bacteria: a — device of Hrabina et al. (2009); b — focus-forming unit of the CSF pathogen adsorbed on pasteurized bacteria; c — genetic material (track 4) of the CSF virus in the bacterial mass of the RR-sediment.

These results indicate a certain probability of an important causal role of the magnetic irradiation factor in the epizootology of the CSF.

B. Study of the virophoria of AD and TD pathogens on pasteurella, streptococci, bifido- and lactobacteria, with epizootological modeling of its consequences on laboratory rodents. The general scheme of the study is shown in Fig. 2.

[‡] BC_{50}/ml — concentration of bacterial cells according to the results of densitometry (by McFarland)

[§] https://www.grander.com/intl-en/international/grander-water/what-is-grander-water

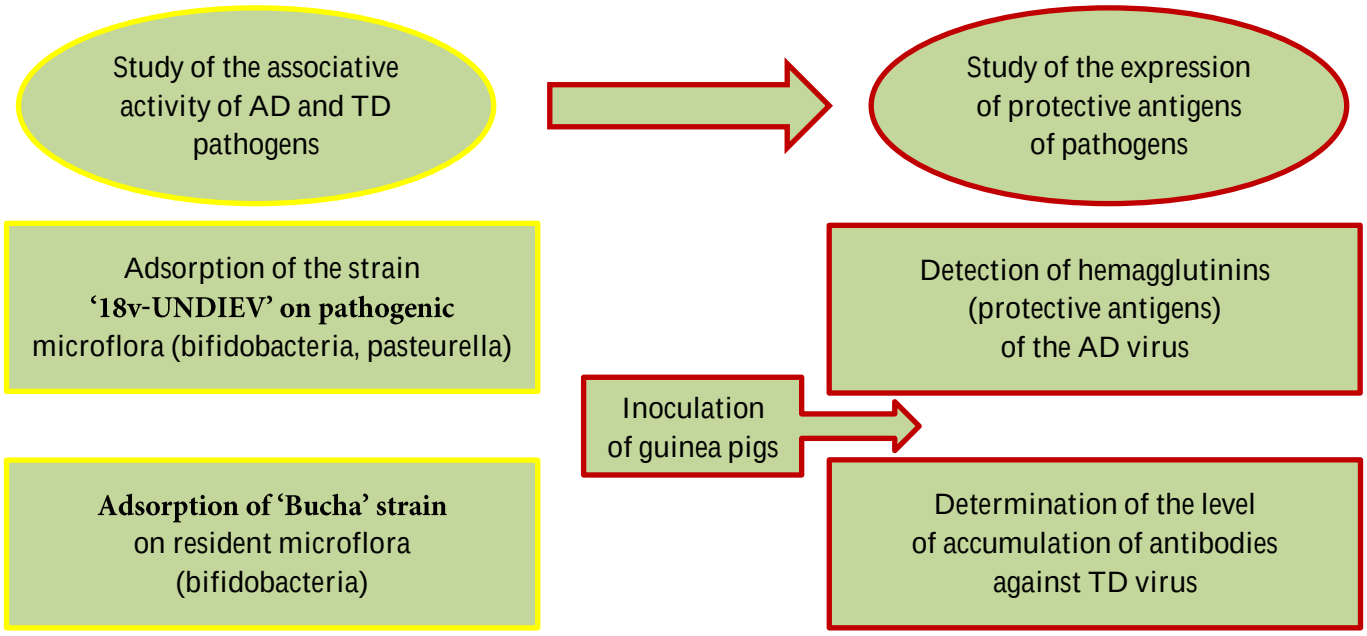


Figure 2. Scheme of the study of bacterial virophoria of the pig microbiome as a factor in the pathogenesis of AD and TD.

The adsorption of AD and TD pathogens on bacteria of the resident and pathogenic microflora of the pig was studied in the context of the study of the associative activity of these infectious agents and its effect on the expression of protective antigens of AD and TD viruses. Table 1 summarizes the results of studies of the intensity of adsorption of the mouse trophovariant strain '18v-UNDIEV' on the reference strains of bacteria of the species *Pasteurella multocida* No. 7, *Salmonella choleraesuis* No. 3–4 (pathogenic microflora), *Bifidobacterium bifidum* and *Lactobacillus casei* (resident microflora) under alkaline (pH 8.5), neutral (pH 7.4) and acidic (pH 3.0) conditions.

Table 1 — Results of the study of adsorption of the strain '18v-UNDIEV' of ADV on bacteria of pathogenic and resident microflora at different acidity of the medium

No.	Samples under test		ADV titer (lg CFU_{50}/ml) in sample fractions at the pH of the corresponding buffer solutions:		
	The kind of sample	Sample fraction	8.5	7.4	3.0
1	Virus suspension	All fractions [1)]	4.5	4.5	4.5
A mixture of ADV strain '8v-UNDIEV' and bacteria of strain No. 7 of *Pasteurella multocida*					
2	Virus-bacterial mixture	Supernatant [2)]	3.5	3.8	2.3
		Sediment [2)]	2.7	4.2	1.0
		adsorption, %	≤ 1.5	≤ 50	0
A mixture of ADV strain '18v-UNDIEV' and bacteria of strain No. 34 *Salmonella choleraesuis*					
3	Virus-bacterial mixture	Supernatant [2)]	3.2	3.8	2.3
		Sediment [2)]	3.8	2.7	0
		adsorption, %	≤ 20	≤ 1.5	0
A mixture of ADV strain '18v-UNDIEV' and bacteria *Bifidobacterium bifidum*					
4	Virus-bacterial mixture	Supernatant [2)]	3.1	3.8	1.0
		Sediment [2)]	4.1	2.7	0
		adsorption, %	≤ 40	≤ 1.5	0
A mixture of ADV strain '18v-UNDIEV' and bacteria *Lactobacillus casei*					
5	Virus-bacterial mixture	Supernatant [2)]	2.8	3.8	2.3
		Sediment [2)]	3.7	2.5	0
		adsorption, %	≤ 15	≤ 1.0	0

Notes: [1)] titer values before the preparation of the mixtures, which are assumed to be 100%; [2)] titer values after preparation and storage of samples, based on the results of 2–3 series of experiments for each parameter ($p < 0.010–0.017$).

It was found that on the bacteria *Salmonella choleraesuis* No. 34, *Bacterium bifidum* and *Lactobacillus casei*, AD virus was adsorbed with an efficiency of 15–45% in the pH range of 8.5–9.5. At a neutral pH (7.4), no more than 1.5% of the virus was adsorbed, and at an acidic pH (3.0), AD virus was not adsorbed at all and was inactivated within 3–4 hours. On bacteria *Pasteurella multocida* No. 7, AD virus was adsorbed in the pH range of 8.5–9.5 with an efficiency of no more than 1.5%; at neutral pH (7.4), up to 50% of the virus was adsorbed, and at acidic pH (3.0), no more than 1.5% of the AD virus was adsorbed.

AD virus adsorbed on pasteurella was inactivated at 37 °C for 4 hours by 0.25 lg TDC_{50}/ml (0.004%), while unadsorbed virus lost 2.25 lg TDC_{50}/ml of infectious activity (0.4%) under the same conditions.

Table 2 summarizes the data on the effect of AD virus adsorption on bifidobacteria on its pathogenicity for mice. It was found that the adsorbed virus does not enter the mouse brain under the experimental conditions ($p < 0.01$), but retains pathogenicity due to damage to parenchymal organs. This may be associated with the selective adsorption of pathogen variants with the indicated tropism to mouse tissues under experimental conditions.

On the other hand, such a model of AD-pasteurellosis comorbidity pathogenesis may result from increased sensitivity of mouse tissues to pasteurella toxins under the influence of the AD pathogen.

Table 3 summarizes the results of the study of the effect of pH on the adsorption of teschovirus on pasteurella, streptococcus and bifidobacterium. These data show that TD virus is actively adsorbed on bacteria only at alkaline pH (8.5). It is most active on streptococci (item 6 of Table 3, $p < 0.01$). The teschovirus adapted to the organism of guinea pigs, adsorbed on bifidobacteria cells, was inactivated by 1.5 lgTCD_{50}/ml (i. e., by 0.001 %) during 5–9 hours of incubation at 50 °C, and its free form — by 4.0–5.0 lgTCD_{50}/ml, i. e., by 42% ($n = 8$, $p < 0.01$). Thus, in the pathogenesis and epizootology of TD, the virophoria of the resident rodent intestinal microflora can be considered a natural mechanism of protection of the pathogen from adverse factors.

The following consequences of teschovirus adsorption on bacteria can be seen in Table 4 — the effect of the latter on the infectious activity of the pathogen in the body of the guinea pig. At a probability level of $p < 0.01$ ($n = 9$), it was found that mice infected with TD virus adsorbed on bifidobacteria at a dose of 6.0 lgTCD_{50}/ml showed no signs of disease, gave normal offspring (not shown in the table) and showed no signs of viral infection (no virus accumulation in target organs and no seroconversion to TD pathogen).

Thus, the interaction of the TD virus with bifidobacteria inhibits viral reproduction in the body of infected guinea pigs and, accordingly, the formation of a persistent infection, which is typical for rodents infected with the free form of the TD pathogen. Virophorous pasteurella and streptococci do not lose their pathogenic properties for rodents. In guinea pigs that survived streptococcal infection from virophorous bacteria ($n = 2$ out of $n = 5$), antiviral antibodies in significant titers were detected 3 weeks after intraperitoneal infection. This indicates that the reproductive activity of teschovirus is preserved in the presence of streptococci, unlike bifidobacteria and pasteurella.

In the context of the above results, it is advisable to present a clinical case of AD in a boar with reproductive disorders, in the semen of which, during the examination, the causative agent of this disease was detected. Initially, a positive result was obtained when staining semen smears according to the protocol for the direct method of fluorescent antibodies against the AD virus (Fig. 3). Then the presence of the virus genetic material was confirmed by PCR.

Based on many years of NSC 'IECVM' experience in studying bacterial virophoria, we 'blindly' sent a sample of primary bacilli from this ejaculate for PCR analysis to another department of our research center. As expected, we received a positive result: the bacterial culture (mainly *Neisseria* and *Pasteurella*) contained the AD pathogen genome. A similar result was obtained during the examination of the ejaculate of a boar carrying the virus from another pig farm (Fig. 3b).

Table 2 — Results of the study of the effect of resident and pathogenic bacteria on the pathogenicity of the AD pathogen for mice

Samples (30% suspensions)	AD virus hemagglutinin titer / its infectious titer (lg TCD_{50}/g)		
	Mice after injection of bifidobacteria (n = 6)	Mice infected with virophorous bifidobacteria (n = 16)	Mice infected with AD virus (n = 12)
24 hours after infection			
of the brain	0 / 0	0 / 0	0 / ≤ 2.0
spleen	0 / 0	0 / ≤ 2.0	1:40–1:80 / ≤ 3.0
96 hours after infection			
of the brain	0 / 0	0 / 0	1:20–1:40 / ≤ 7.0
spleen	0 / 0	1:40–1:320 / ≤ 5.0	1:80–1:320 / ≤ 7.0
Time / percentage of mouse deaths			
	0 / 0	5–9 days / 43.8%	2–3 days / 91.7%

Note: [1)] intranasal infection.

Table 3 — Results of the study of adsorption of TD virus strain 'Bucha' on bifidobacteria, pasteurella and streptococci at different pH

No.	Samples under test		ADV titer (lg TCD_{50}/ml) in sample fractions at the pH of the corresponding buffer solutions:		
	The kind of sample	Sample fraction	8.5	7.4	3.0
Adsorption on *Bifidobacterium bifidum*					
1	Virus suspension	All fractions [1)]	6.5	6.5	6.5
2	Virus-bacterial mixture	Supernatant [2)]	4.8	4.8	6.0
		Sediment [2)]	5.8	2.7	0
		adsorption, %	≤ 20	≤ 10	0
Adsorption on *Pasteurella multocida*					
3	Virus suspension	All fractions [1)]	6.5	6.5	6.5
4	Virus-bacterial mixture	Supernatant [2)]	5.0	6.0	6.0
		Sediment [2)]	3.5	0	0
		adsorption, %	≤ 10	0	0
Adsorption on *Streptococcus suis*					
5	Virus suspension	All fractions [1)]	6.5	6.5	6.5
6	Virus-bacterial mixture	Supernatant [2)]	4.5	5.0	6.0
		Sediment [2)]	6.5	3.0	0
		adsorption, %	≤ 80	≤ 10	0

Notes: [1)] titer values before the preparation of the mixtures, which are assumed to be 100%; [2)] titer values after preparation and storage of samples, based on the results of 3 series of experiments for each parameter ($p < 0.010–0.017$).

Table 4 — Results of the study of the effect of bacteria of the pig microbiome and guinea pigs on the reproductive activity of the TD pathogen in guinea pigs

No	10–30% of the suspension of target organs in guinea pigs	Characteristics of infected guinea pigs [1)]		
		Virus titer TD (lg TCD_{50}/ml)	Death of infected guinea pigs	Virus-neutralizing antibody titer
Intraperitoneal infection with a viral suspension of the 'Bucha' strain				
1	duodenum	0 (n = 4)	0 (n = 4)	1:4–1:8 (n = 4)
2	ileum	1.0–3.0 (n = 4)		
Intraperitoneal infection with virophorous bifidobacteria				
3	duodenum	0 (n = 5)	0 (n = 5)	0 (n = 5)
4	ileum	0 (n = 5)		
Intraperitoneal infection with virophorous pasteurella				
5	duodenum	0 (n = 4)	75% (n = 4)	0 (n = 1)
6	ileum	1.0–1.5 (n = 4)		
Intraperitoneal infection with virophorous streptococci				
7	duodenum	1.0–1.5 (n = 5)	60% (n = 3)	1:8–1:32 (n = 2)
8	ileum	3.0–3.5 (n = 5)		

Notes: [1)] recording within 21 days after infection.

This gives reason to believe that the semen of boars carrying the virus is contaminated not so much with the AD virus as with virophorous bacteria, which, in turn, adsorbed on spermatozoa. After all, the AD pathogen is neurotropic: although it affects almost all organs, in all cases it affects only their nervous tissue (Fan et al., 2019), which is not present in sperm. Nevertheless, microbes, including virophorous ones, are actively adsorbed on spermatozoa, as shown in Fig. 3.

Discussion *(Substantiation of the doctrine of transfer the epizootic process of dangerous viral infections to the enzootic one and their rooting in pig production through the integration of their pathogen with the pig microbiome in the form of comorbid viral and bacterial infections).* Back in the 1960s, Prof. Kulesko and his students developed a method of immunization of wild and domestic pigs against CSF based on the virophoria of its pathogen on bacteria of the vaccine strain of erysipelas.

Due to the tropism of erysipelas bacteria to the tonsils, oral vaccination with this drug practically saved the wild boar population of the Belovezhskaya Pushcha Reserve from imminent death (Kulesko and Lichtman, 1961).

Since then, the NSC 'IECVM' has established a tradition of researching and analyzing animal infectious pathology and epizootology in the format of biocenology — from the angle of interaction of viruses and bacteria with each other and their biological hosts, taking into account the influence of environmental factors. In the late 1980s, discussions between scientists from Ukrainian Research Institute of Experimental Veterinary Medicine (former name of the NSC 'IECVM') and Research Agricultural Institute of the Ministry of Agriculture of USSR (Gvardeyskiy, Kazakhstan) began on the virophoria of bacteria with certain types of herpes-, morbilli-, pox-, picorna- and rhabdoviruses, the staff of the institute formed and developed a powerful school of studying the epizootology of associated viral and bacterial infections in cattle (Fuks, 1993, 1994, 1999; Fuks et al., 1993, 1994).

One of the achievements of this school was undoubtedly the establishment of the phenomenon of reproduction of herpesvirus of infectious rhinotracheitis in *Bacillus alvei* — bee bacteria (Fuks, 1994). Already in the period 2015–2022, during epizootic surveys and research on relevant academic topics, we often observed the phenomenon of bacterial virophoria in the analysis of samples of pathological material, semen and feed from pig facilities contaminated with microbial associations containing PRNI pathogens: circoviruses, parvo-, teschoviruses, Aujeszky's disease and PRRS viruses, smallpox and PED. These unpublished data are fully consistent with the hypothesis of Katharina Ribbeck from Harvard University that viruses use bacteria and sperm as a factor in the transmission of viral infections in humans and animals (Ribbeck, 2009). Only after systematizing our similar epidemiological data on comorbid infections with African swine fever virus in the StopAfSFVmix doctrine, we came to the conclusion that the 'biocenological melting pot' of the above-mentioned associated reproductive and neonatal infections of pigs is a natural tool for transfer the acute epizootic process of monoinfections of pathogens exotic to pig production to the enzootic process of PRNI. Such infections were certainly CSF, AD, PCV-2, porcine parvovirus, PRRS and PED, which now fall under the definition of PRNI. In our opinion, unfortunately, the situation with ASF is developing in the same way.

In Fig. 4a, we summarized the PRNI enzootic chain, which is fully consistent with and, in our opinion, clarifies the French-American doctrine of the establishment of emergent pathogens (Morse, 1995; Desenclos and De Valk, 2005) in new nosoarea territories (Fig. 4b). Our refinement concerns the final node of the chain of establishment of an exotic infectious agent (Fig. 4b, c).

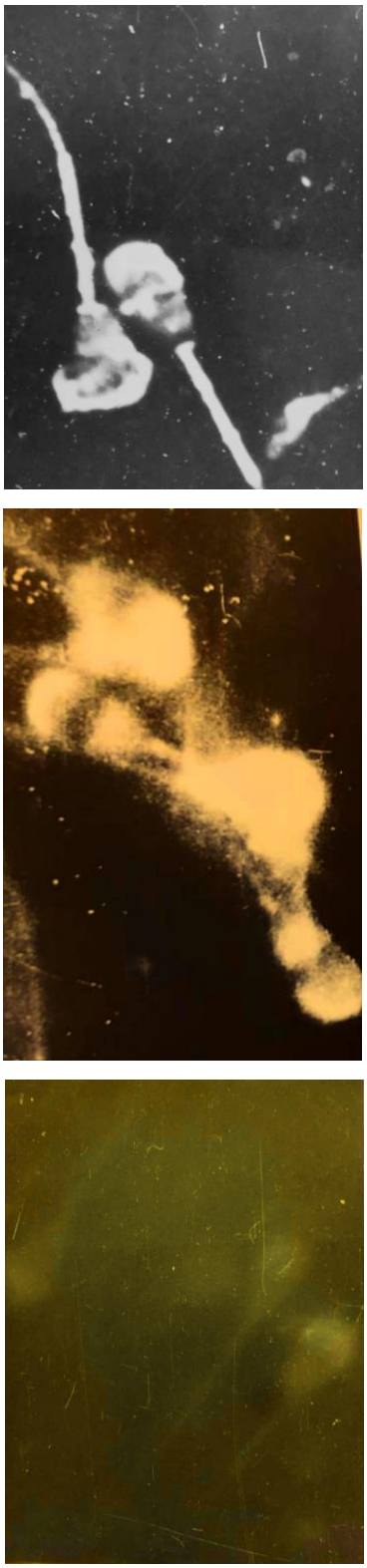


Figure 3. Results of examination of ejaculate swabs from boars No. 1 (a and c) and No. 2 (b) by direct fluorescent antibody method for AD virus carriage. In photos a and b — native smears (ethanol fixation), in photo c — a smear of ejaculate from boar No. 1, which before ethanol fixation was exposed for 15 min in a buffer solution with a pH 3.0 to elute virophorous bacteria from sperm.

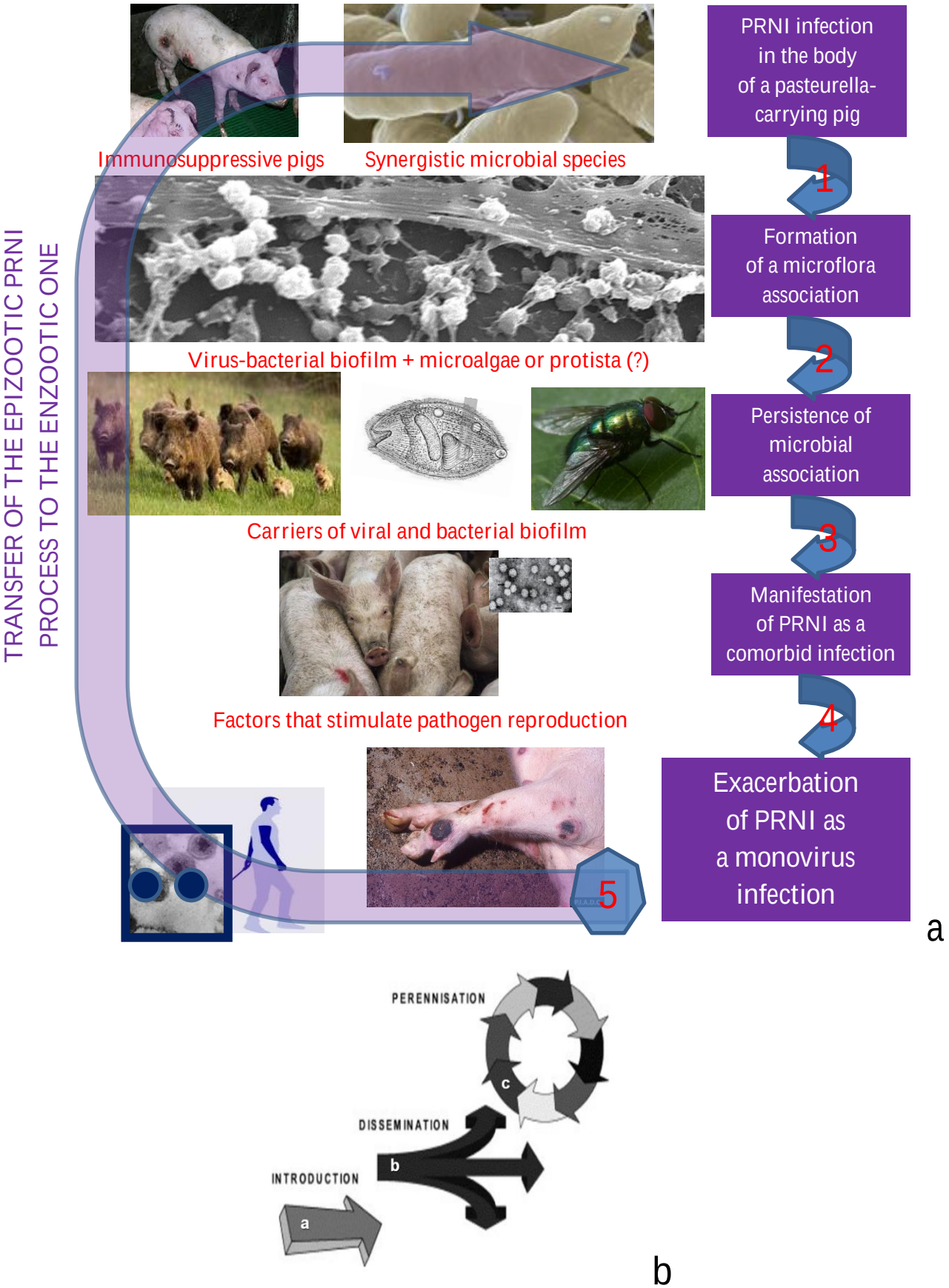


Figure 4. Determination of the role of bacterial virophoria as a key element in the formation of stationary PRNI foci (a) and in transfer acute phases of the enzootic process to chronic/comorbid ones (b, node 'c').

The 'Perennization' node (Fig. 4b, c) of the epizootic chain of emergent infections schematically summarizes the enzootic cycle of gradual exacerbation (dark arrows in shades from black to light gray) and 'normalization' of the epizootic situation (white arrow). This pattern is also characteristic of PRNI. Therefore, our concept (Fig. 4a) explains the interaction of an infectious agent new to a given pig herd with the pig's microbiome and the environment of a given territory/stationary focus. This interaction can lead to the elimination of the exotic agent and then the epizootic process will not become enzootic (Fig. 4b, b). If the agent 'finds a partner' in the above microbiome, it can be temporarily stored in microbial biofilms in the pig's body or in the environment, which at stage 2 (Fig. 4a, arrow 2) during the formation of a stationary enzootic focus can enter ecological niches in various ways to interact with microbially fed protists cells and other reservoir hosts of the pathogen. Under the influence of environmental factors (solar activity, weather factors, etc.) and the pig's body (e. g., immunosuppression factors), the target agent is activated (Fig. 4a, arrow 3), which leads to an exacerbation of the epizootic situation in the form of comorbid PRNI — different levels of intensity (Fig. 4b, c). In the next phase of the enzootic cycle (Fig. 4a, arrow 4), the target agent acquires, according to its inherent nature, a sufficient level of pathogenicity for the pig and is clinically manifested as a corresponding monoinfection, which corresponds to the black arrows in Fig. 4b, c.

We realize that there are many 'white spots' in our model of the rooting of emergent infectious agents in the PRNI microbiome, but the key importance of bacterial virophoria for triggering the scenario of transfer the epizootic process of emergent porcine viruses to an enzootic one is obvious. We see further development of the doctrine of transfer the epizootic process of dangerous viral infections to the enzootic one in the study of the possible persistence of PRNI pathogens in the protozoa (especially in their cysts), as well as in the study of the nature of the increase in the virulence of infectious agents of the pig in stationary foci of PRNI.

Conclusions. 1. The rooting of dangerous viral infections (Aujeszky's and Teschen's diseases, circovirus and parvovirus infections, reproductive and respiratory syndrome, and endemic porcine diarrhea) in pig production has always been accompanied by the 'engraftment' of their pathogens in the microbiome of pig production facilities in the form of comorbid (i. e. clinically manifested) and/or associated infections (i. e. similar to the group of Minimal Residual Human Diseases — Maladie Résiduelle Minimale, MRD).

2. The key role in the rooting of these diseases and the formation of their stationary foci in pig production is played by the virophoria of bacteria synergistic with their pathogens, in particular, as part of the etiological microflora of reproductive and neonatal infections in pigs.

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RISK OF SWINE INFLUENZA FOR VETERINARY MEDICINE AND HUMAN HEALTH IN UKRAINE

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Summary. The article provides a brief review of the literature on influenza viruses, including their prevalence, classification, pathogenicity, ability to mutate and reassort, and some peculiarities of their biological properties. Special attention is given to swine influenza, since interspecies transmission of influenza viruses between pigs and humans can have serious consequences for both human and veterinary medicine. The results of pilot studies conducted at the NSC 'IECVM' prove the circulation of influenza A viruses among pigs in Ukraine — 324 samples of blood sera from pigs of different age groups from different regions of Ukraine were tested using an ELISA test system, 48 (14.8%) positive samples were found. Seroprevalence reached 100% in some farms and antibodies were detected in animals aged 24–73 days. The prospect of further work is to conduct surveillance studies (serological, molecular biological, virological) of pigs in both large breeding complexes and private households to detect antibodies to influenza A and, based on the results obtained, to draw conclusions about the circulation of influenza A viruses in Ukraine

Keywords: monitoring, swine flu, influenza viruses, enzyme-linked immunosorbent assay

Introduction. Today, the issue of zoonotic pathogens has become particularly relevant following the pandemic of animal-borne coronavirus infections. Of particular concern are avian influenza viruses, swine influenza, and coronaviruses, which can cause severe morbidity and mortality in humans (Coker et al., 2011a; Coker et al., 2011b). According to the WHO, influenza epidemics and pandemics are among the ten worst epidemics and pandemics in recent human history caused by zoonotic viral agents, along with diseases such as Marburg, Ebola, Nipah, MERS, SARS-CoV2. In particular, influenza viruses have caused at least four confirmed pandemics — 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1) (Piret, and Boivin, 2021; Easterday, 2003; Krueger and Gray, 2012; Scholtissek et al., 1978). Despite its long history, influenza remains one of the most dangerous and unpredictable infectious diseases in the world. To date, influenza A viruses are considered one of the greatest threats for the next global pandemic due to the peculiarities of the pathogen's structure, the large number of natural reservoirs and hosts of the virus, and the ability of the virus to move beyond the natural reservoir and cross the interspecies barrier to infect other species, including humans.

The ecology of influenza A viruses is complex and includes a wide range of host species in birds and mammals, such as human influenza virus (hIV), swine influenza virus (swIV), avian influenza virus (AIV), equine influenza virus (EIV), canine influenza virus (CIV), and bat influenza virus (Muramoto et al., 2013; Shi et al., 2014; Yamayoshi et al., 2016; Suarez, 2016).

The current classification of influenza viruses is based on subtypes of surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N9). It is believed that influenza in humans is caused by viruses containing subtypes H1, H2, H3 and N1, N2, although cases of human infection with other influenza virus subtypes are reported. Avian influenza viruses include viruses with subtypes H1 to H16 and N1 to N9, while H17N10 and H18N11 have so far been detected only in bats (Suarez, 2016; Gamblin and Skehel, 2010).

Influenza viruses have a high mutation rate and are constantly changing, which allows them to adapt quickly to environmental changes, such as interspecies transmission. The rapid evolution of influenza viruses is the result of two mechanisms: reassortment (antigenic shift) and point mutations (antigenic drift) (Shao et al., 2017). Both mechanisms play a key role in the emergence of new influenza viruses capable of crossing the host barrier. Once the virus enters the body of a new host, it must adapt and change in order to spread in the new population (Nelson et al., 2014; Lewis et al., 2016; Rajao et al., 2018).

Of all animals and birds, pigs can play a major role in the amplification and creation of new viruses, including influenza (Krueger and Gray, 2012; Mena et al., 2016; Pepin, Miller and Wilber, 2021; Pickering et al., 2021; Wardeh, Baylis and Blagrove, 2021; Hennig et al., 2022).

Transmission between pigs usually occurs through close contact between animals and contaminated objects. Although the severity of the disease is influenced by many factors, including the virus strain, the disease usually begins suddenly as an acute respiratory illness — asymptomatic or mild fever, coughing, sneezing, nasal and ocular discharge, shortness of breath, inactivity and decreased appetite (Rajao et al., 2014; Rajao and Vincent,

2015). There is high herd morbidity (approximately 100%) and usually low mortality (< 1%), but the disease causes high economic losses due to reduced weight gain, especially in young animals (Vincent et al., 2008). The incubation period of the disease is 1–3 days, and recovery begins 4–7 days after disease onset. Virus replication is usually limited to the epithelial cells of the respiratory tract, especially the nasal mucosa, tonsils, trachea, and lungs (Abdelwhab and Mettenleiter, 2023). However, the main concern about swine flu is not so much the economic consequences for the swine industry, but the risks of a new pandemic virus.

Several subtypes of influenza A viruses circulate in swine, including H1N1, H1N2, H3N1, and H3N2 (Lewis et al., 2016; Brockwell-Staats, Webster and Webby, 2009; Chauhan and Gordon, 2020). The classical 'swine' H1N1 virus was first isolated from a pig in the United States in 1930 (Shope, 1931), although an influenza-like disease was clinically recognized in the second half of 1918 during the so-called 'Spanish flu' epidemic (Koen, 1919; Hinshaw et al., 1978). The origin of this pandemic virus — from humans to swine or vice versa — is still unclear (Reid and Taubenberger, 1999; Webster, 1999), but the human virus of 1918 and the swine viruses of 1930 are closely related (Taubenberger et al., 1997; Reid et al., 1999).

Most swine influenza viruses are reassortants, combining genes from swine, avian and human viruses. This confirms the basic dogma that pigs can act as a 'mixing vessel' between human and avian influenza viruses (Wardeh, Baylis and Blagrove, 2021; Hennig et al., 2022; Abdelwhab and Mettenleiter, 2023). This is mainly due to the fact that pigs have both avian and human type receptors (α-*2.3* and α-*2.6*) and can be infected with both avian and human influenza viruses (Nelli et al., 2010). However, it is not entirely clear whether pigs are more susceptible to avian virus infection than humans (Rajao, Vincent and Perez, 2019), although transmission of swIV to poultry, mainly turkeys, has been reported (Choi et al., 2004; Reid et al., 2012; Berhane et al., 2016).

The influenza virus that caused the 1918 pandemic has remained relatively antigenically stable for eight decades without causing major problems for pig farms. The pathogen first entered the European continent in 1976 with exported animals from the United States to Europe and spread rapidly among pigs. And in 1979, a new virus was identified in Europe, which originated from wild ducks and was antigenically different from the 'classic' swine A (H1N1) strain (Zell, Scholtissek and Ludwig, 2012). This 'avian' strain quickly and completely replaced the 'classical' swine influenza viruses circulating on the European continent and also spread in Asia. The classic swine H1N1 and avian H1N1 viruses were the main ones circulating in pigs until the 1990s. In the 1980s, new reassortant H1N2 viruses were discovered in England and then in Europe, which gave rise to new strains of swine influenza virus (Brown et al., 1998).

A new triple reassortant virus with seasonal human H3N2 surface genes emerged in North America in the late 1990s (Olsen, 2002) and subsequently reassortment with classical viruses (H1N1, H1N2) and the creation of a new H3 lineage (H3N2) (Bakre et al., 2021; Karasin et al., 2002; Webby et al., 2004).

In Europe, the H3N2 virus of 'human' origin, derived from the 1968 pandemic virus, was introduced in the 1980s. This virus became widespread after reassorting with the avian H1N1 virus, which was introduced into European pigs in 1979 and remains endemic today (Castrucci et al., 1994; Simon et al., 2014).

Another virus, H1N2, was discovered in 1994 and contained H1, derived from the 1980 seasonal human H1N1 influenza virus, and N2, distinct from the previously isolated H3N2 virus. This virus acquired the internal gene set of the 1979 avian virus through recombination and is now also endemic in Europe (Lewis et al., 2016; Brown et al., 1998; Marozin et al., 2002).

In 2009 these viruses recombined again, this time with influenza A viruses from the Eurasian swine flu lineage. As a result, the strain known as pandemic influenza virus or A/H1N1pdm09 spread rapidly around the world, sickening millions of people (Hennig et al., 2022).

As noted above, interspecies transmission of influenza viruses between pigs and humans is believed to have occurred since the 1918 pandemic and is at least partially responsible for the 2009 swine-origin human influenza, highlighting its zoonotic potential (Hennig et al., 2022; Reid and Taubenberger, 1999; Webster, 1999).

The exchange of viruses between humans and pigs in most cases occurs through close contact, especially on pig farms or slaughterhouses, which is an important risk factor for swine flu infection among pig farm workers. (Borkenhagen et al., 2020; Ma et al., 2018; El Zowalaty et al., 2022; Chauhan and Gordon, 2022). Due to the industrialization of pig production, dense populations of pigs and humans remain in close proximity, and this can increase the risk of transmission of influenza viruses. Zoonotic transmission of H1N1 viruses from pigs has been reported repeatedly in the United States (Hinshaw et al., 1978; Wentworth et al., 1997; Dacso et al., 1984), Europe (Jong et al., 1988; Andersen et al., 2022), Asia (Li et al., 2019; Yang et al., 2022), New Zealand (Eason and Sage, 1980) and Australia (Deng et al., 2020), and in some cases, deaths of infected people have been recorded (Smith et al., 1976; Top and Russell, 1977; Patriarca et al., 1984; Rota et al., 1989; Kimura, Adlakha and Simon, 1998; Wentworth et al., 1994). Thus, from 2010 to 2021, about 700 confirmed cases were reported worldwide, most of which occurred in children or immunocompromised patients (Hennig et al., 2022). Interestingly, transmission of the influenza virus from pig

to human is regularly reported, but the number of human infections is much lower than in pigs (Freidl et al., 2014).

Human influenza is rarely recorded in swine; usually these viruses are reassorted and reappear, retaining only some segments of viral genes of human origin, often with marked genetic differences from the ancestral strain (Nelson et al., 2014; Lewis et al., 2016; Rajao et al., 2018).

It should be noted that since 1918, all pandemic human influenza viruses, with the exception of the 1958 H2N2 virus, have been transmitted by zoonotic means — from humans to swine populations (Hennig et al., 2022). Human influenza viruses have been regularly isolated from swine in the United States, Europe, Asia, and Australia (Castrucci et al., 1994; Deng et al., 2020; Shortridge et al., 1977; Shortridge, Cherry and Kendal, 1979; Nakajima et al., 1982; Katsuda et al., 1995; Nelson et al., 2015a). In addition, anthropozoonotic transmission of seasonal and pandemic influenza viruses to swine has resulted in the creation of a long-term reservoir of zoonotic influenza viruses in swine (Kessler et al., 2021; Glud et al., 2021). In addition to the 'human' subtypes of the virus, the circulation of other subtypes such as H5, H7 and H9 has been reported in pigs (Hennig et al., 2022).

According to some reports (Sikkema et al., 2016), the frequency of subclinical infections in livestock workers ranges from 15% to 40%. And only vaccination programs for workers against seasonal influenza can prevent the occurrence and transmission of infections from animals or vice versa (El Zowalaty et al., 2022).

Swine flu, one of the most prominent zoonotic infections, illustrates the role of trade in its global spread (Nelson et al., 2015b; Gcumisa, Oguttu and Masafu, 2016). In most cases, this is true for regions such as Southeast Asia, where more than 50% of the world's pork production is produced in enterprises of various types (pig farms, households) (Gale, 2017).

Thus, in most cases, small-scale pig farms are characterized by a lack of highly qualified personnel, uncontrolled movement of breeding animals and lack of quarantine measures, intensive and frequent contact between humans and different animal species. All this increases the risk of virus spread between pigs and service personnel, as well as the emergence of new infectious diseases, including zoonotic diseases with pandemic potential (Trevennec et al., 2011; Baudon et al., 2017).

Large industrial swine farms, commonly known as indoor animal feeding operations, are also believed to be sites of origin and transmission of various pathogens, such as porcine reproductive and respiratory syndrome virus, porcine epidemic diarrhea virus, coronavirus acute diarrhea syndrome, and African swine fever (massive outbreak in 2018–2019) (Borkenhagen et al., 2020). For influenza, the importation of livestock in China and other Asian countries has led to the co-circulation of both European (or Eurasian) and North American triple reassortant virus strains containing genes of human origin (Nelson et al., 2015b; Poonsuk et al., 2013).

In addition, reassortant genotypes between these strains containing HA and/or NA genes from human H1N1 and H3N2 viruses have been detected in Asia since the 1960s and have become widespread in swine (Nelson et al., 2014; Liang et al., 2014; Cheung et al., 2023).

Although there have been numerous reports on the prevalence of IAV in organized (commercial) pig farms worldwide during the twentieth century (Chauhan and Gordon, 2020), surveillance of influenza A in pig populations was ignored until the outbreak of the swine flu pandemic in March 2009 (Mena et al., 2016). This appears to have served as a catalyst for surveillance, as most studies were initiated in 2009 (Chumsang et al., 2021, Bravo-Vasquez et al., 2020; Gonzalez-Reiche et al., 2017).

The special role of swine influenza has long been recognized, but these viruses remain highly mobile targets that are notoriously difficult to diagnose due to their remarkable genetic flexibility. One of the main diagnostic tools is molecular genetic testing, which is increasingly replacing traditional laboratory methods. These methods, which use nucleic acid amplification, are characterized by high sensitivity, specificity, and less stringent requirements for the biological material to be tested (El Zowalaty et al., 2022; Chauhan and Gordon, 2022; Gonzalez-Reiche et al., 2017; Mahardika et al., 2018). In most cases, nasal secretions from animals are used as the test sample for molecular studies (Decorte et al., 2015; Janke, 2014). The polymerase chain reaction method provides reliable results in a short period of time, but due to the simultaneous circulation of several influenza virus subtypes, identification errors may occur (Abdelwhab and Mettenleiter, 2023). Consequently, if the virus concentration in samples with these specific subtypes is low, these samples may be misidentified and the subtype with the lower concentration may not be successfully detected (Blair et al., 2019). According to the literature, misidentification also occurs for H3N2 and H1N1 swine subtypes. This is due to the fact that both viruses have genetic similarities with the seasonal human H1N1pdm09 virus (sharing some common gene segments such as M, NS, NP, and HA) (Taylor et al., 2019).

Serologic tests are crucial for the diagnosis of clinical disease, for the immune profile of the herd, for determining the timing of vaccination, for monitoring the effectiveness of vaccination, and for epidemiological studies to identify the dominant virus serotype. Modern serological tests include the hemagglutination inhibition test (HIT) and the enzyme-linked immunosorbent assay (ELISA). ELISA is the most popular serologic test for the diagnosis of swine influenza due to its simplicity, wide range of available tests, low cost and screening nature

(Bravo-Vasquez et al., 2020; Osoro et al., 2019; Jimenez-Bluhm et al., 2018). However, it should be noted that this reaction only detects antibodies to specific subtypes and does not provide complete information on all genotypes circulating in the herd (Chauhan and Gordon, 2022). The ELISA can effectively detect antibodies to swine influenza virus in the serum of animals seven days after exposure to the pathogen or vaccination with a prophylactic vaccine, while the peak may be reached in two to three weeks (Van Reeth, Labarque and Pensaert, 2006; Larsen et al., 2000).

However, it is important to remember that serological tests can produce false-positive or false-negative results, so it is necessary to send material from a large number of animals to obtain reliable test results. In addition, the test systems detect specific antibodies homologous to the antigens in the test system, and all are of little use in detecting antibodies to heterologous influenza viruses.

There is no current information on the epidemiological situation of swine flu in Ukraine. There are only some reports on the presence of antibodies to influenza A virus in wild boars (up to 22.5% of positive samples were detected) (Kovalenko et al., 2017). Therefore, the aim of our study was to perform pilot studies on blood sera of pigs of different age groups from different farms for the presence of antibodies to influenza A viruses.

Materials and methods. The study was conducted at the Department of Poultry Diseases of the NSC 'IECVM'. Blood sera from pigs were collected according to generally accepted methods. For analysis, 324 samples of blood sera from pigs of different ages from farms located in different regions of Ukraine were selected. The sampling scheme is presented in Table 1.

Table 1 — Sampling of blood sera from pigs in different years

Year of sampling	Region	Total number of involved farms	Total number of samples collected
2015 (archive samples)	Poltava	6	60
2021	Poltava	2	119
	Zaporizhzhia	2	14
2023	Khmelnytskyi	1	15
	Dnipropetrovsk	3	75
	Zaporizhzhia	1	15
	Sumy	1	16
	Lviv	1	10
Total			324

The study for the presence of antibodies to influenza viruses was carried out by ELISA using kits manufactured by IDEXX Influenza A Ab Test (USA) and ID Screen® Influenza A Antibody Competition Multi-species (France). All studies were performed according to generally accepted methods in compliance with all biosecurity and biosafety requirements.

Results. The results of serologic testing of samples collected in 2015, 2021, and 2023 are presented in Tables 2–4.

Table 2 — ELISA results for the presence of antibodies to influenza A in pigs from farms in Poltava Region in 2015

No. farm	Number of samples tested	Techno-logical area	Posi-tive	Nega-tive	Sero-preva-lence, %
1	5	sows	0	5	0
	5	fattening	0	5	0
2	10	fattening	0	10	0
	10	sows	3	7	30
3	5	fattening	0	5	0
4	5	fattening	4	1	80
5	7	boars	4	3	57.1
	4	fattening	2	2	50
6	2	sows	0	2	0
	7	fattening	3	5	42.9

Table 2 shows that 30% of the sows from farm 2 were seropositive for influenza A, while no antibodies to this pathogen were detected in the fattening pigs. In the blood sera of fattening pigs from farms 4–6, antibodies were present in 43% to 80% of the animals, and 57.1% of boars from farm 5 were seropositive for the virus.

Table 3 — ELISA results for the presence of antibodies to influenza A in pig blood sera, 2021

No. farm	Number of samples tested	Techno-logical period/ group	Posi-tive	Nega-tive	Sero-preva-lence, %
Poltava Region, XII-139–21					
1	99	fattening	1	98	1
2	10	growing	0	10	0
	10	fattening	0	10	0
Zaporizhzhia Region					
3	5	sows	4	1	80.0
4	9	sows	2	7	22.2

Analyzing the results presented in Table 3, it can be concluded that only one blood serum sample was positive for influenza in the Poltava Region, and 22.2% and 80% of positive samples were found in sows, i. e. animals older than one year, in two farms in the Zaporizhzhia Region.

Table 4 — ELISA results for the presence of antibodies to influenza A in pig blood sera, 2023.

No. farm	Number of samples tested	Technological period/ age of animals	Positive	Negative	Seroprevalence, %
Khmelnytskyi Region					
1	5	24 days	1	4	20
	5	45–49 days	0	5	0
	5	73 days	2	3	40
Dnipropetrovsk Region					
2	5	24 days	0	5	0
	5	45–49 days	0	5	0
	5	73 days	0	5	0
3	5	24 days	0	5	0
	5	45–49 days	0	5	0
	5	73 days	0	5	0
4	5	24 days	2	3	40
	5	45–49 days	3	2	60
	5	73 days	0	5	0
Zaporizhzhia Region					
5	5	24 days	0	5	0
	5	45–49 days	2	3	40
	5	73 days	5	0	100
Sumy Region					
6	16	growing	0	16	0
Lviv Region					
7	10	fattening	10	0	100

As shown in Table 4, 101 samples of pig blood sera from farms in five regions of Ukraine were tested in 2023, and in each region, animals of all ages were found to be positive for influenza A, with seroprevalence ranging from 20 to 100%.

Conclusions. Analyzing the results of our serological studies of unvaccinated pigs of different age groups, it can be stated that most of the animals positive for influenza were older than 3 months (fattening groups, sows, boars, seroprevalence 20–100%). However, antibodies were also detected in blood sera collected in 2023 in animals aged 24–73 days (15 positive samples out of 75 tested), and in the Zaporizhzhia region, 40 and 100% of samples from animals aged 45–49 and 73 days, respectively, were positive.

This is a very high rate of seropositivity to influenza A among pigs. For example, in Asian countries (China, India, Bangladesh, Bhutan, etc.), the average seropositivity from 2009 to 2021 was 18.28% (25.49%, 19.83%, 12.22%, and 7.74%, respectively). A more thorough typing of the positive samples isolated during 2009–2021 using the HIA showed the following proportions: H1N1 (n = 9, 24.32%); pandemic A/H1N1pdm09 (n = 7, 18.92%); H3N2 (n = 6, 16.22%); H5N1 (n = 2, 5.41%); H5N8 (n = 1, 2.7%); H1N2 (n = 1, 2.7%) (Chauhan and Gordon, 2022).

Therefore, domestic pig populations should not be considered as the only or most important reservoir of potentially zoonotic influenza A virus worldwide. Regular and thorough surveillance of pig populations for influenza A virus is essential to monitor the evolution of swIV. The emergence of influenza A virus in pigs during the last human pandemic in 2009 highlighted the major risks for pig populations in which influenza A virus circulates freely and can trigger zoonotic and anthropozoonotic transmission that endangers human health. In addition, understanding the mechanisms associated with host range specificity and adaptation to pigs will allow us to assess the risks associated with the introduction of new viruses into the pig population, and determining the antigenic characteristics of strains circulating in a particular area will ensure the accurate selection of representative vaccine strains to protect the industry.

The prospect of further research is to conduct monitoring studies (serological, molecular biological, virological) of pig herds in both large breeding complexes and private households to detect antibodies to influenza A and, based on the results obtained, to draw conclusions about the circulation of influenza A viruses in Ukraine. The relevance of the planned work is reflected in our pilot serological studies and the high seropositivity of pigs to influenza A (up to 100%).

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Contents

Part 1. Veterinary medicine

Part 2. Biotechnology

Part 3. Biosafety