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New journal 'Journal for Veterinary Medicine, Biotechnology and Biosafety", discovered in 2015, aimed to consolidate and share the new developments and achievements in the area of biological science. This was recognized as the profile edition for veterinary medicine doctors and biologists in Ukraine. Our journal promotes the research of Ukrainian institutions, publishing their achievements in English, and sharing it among the scientific community. It includes cooperative veterinary and medical aspects, fitting to One Health Approach declared by WHO, OIE, and FAO. It was included in Index Copernicus and eLibrary scientific databases.

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

Sincerely yours,
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Editorial Board Address:
NSC 'Institute of Experimental and Clinical Veterinary Medicine'
83 Pushkinska Str., Kharkiv, Ukraine, 61023
tel. +38 (057) 707-20-53, 704-10-90
E-mail: nsc.iecvm.kharkov@gmail.com, inform@vet.kharkov.ua

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EXPERIMENTAL-CLINICAL ANALYSIS OF SOME ASPECTS OF THE CORONAVIRUSES EMERGENCE IN PIGGERY DURING 1987–2020

Buzun A. I., Stegniy M. Yu., Bobrovitskaya I. A.

National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: epibuz@ukr.net

Summary. The paper presents an analysis of own clinical and experimental data on the participation of ducks in the possible emergence of swine alpha-coronavirus — a virus of endemic diarrhea (PED-CoV), as well as of the porcine beta-coronavirus threats (hemagglutinating encephalomyelitis virus, PHE-CoV) in the COVID pandemia context. The coronavirus of duck enteritis (DE-CoV) was studied in the nineties of the twentieth century: biodiversity of its morphology includes the morphological variants identical to the morphology of PED-CoV and PHE-CoV. Moreover, hemagglutinins of all these viruses have a bilateral affinity among them on the level 24–42%. Obtained data suggest the real risk of ducks' participation in the emergence of at least alpha-coronavirus infections in pigs. There are also threats of the emergence of porcine beta-coronavirus infection under the influence of COVID-19 in industrial pig herds

Keywords: duck enteritis, porcine epidemic diarrhea, porcine hemagglutinating encephalomyelitis, electron microscopy

Introduction. The modern world practice of pig breeding in the last 40–30 years has made significant progress in regulating the epidemic process of coronavirus etiology. Experience in this area has crystallized in the collision with four coronavirus infections. It is summarized and widely highlighted in the specialized periodicals on pig breeding as are presented in Hancox (2020):

Disease	Known as	Clinical signs	Notes
Porcine epidemic diarrhea	PED	• Watery diarrhea and vomiting • Rapid spread • 100% mortality in pigs < 7 days old	If you see these clinical signs contact your vet immediately
Transmissible gastroenteritis	TGE		
Hemagg-lutinating encephalo-myelitis virus	Vomiting and wasting disease	• Often no symptoms when infected • Suckling pigs may show vomiting, anorexia, and wasting	Clinical disease is rarely seen
Porcine respiratory coronavirus	PRC	• Often no symptoms	Virus is widespread and antibodies to it protects against TGE

The COVID-2019 pandemic attracted special attention to swine coronaviruses. On the one hand, it posed a direct threat to the pig business through quarantine measures that affected pork purchase (Conklin, 2020).

On the other hand, the pandemic was alarming given the possibility of human coronavirus survival in animal's populations and contra versa (Pennisi, 2020; Komisarenko, 2020).

The Italians were the first to feel this alarm (including the threats from swine) after the COVID-2019 outbreak in northern Italy (Leopardi, Terregino and Paola, 2020).

This is quite justified because the biological host range' expansion is strictly correlated with the emerging potential of viruses (Zhao et al., 2019).

Besides, the new (for 1980) duck coronavirus of Chinese origin, as it was known to a narrow circle of former Soviet experts (Knyazev, 2011), in 1990 became the main cause of the collapse of the duck industry in some republics of the former USSR (see Discussion). Below we will try to analyze some of our own experimental and clinical data on duck and porcine coronaviruses, obtained in various scientific teams in 1989–2020 in the aspect of current threats for piggery.

Materials and methods. Coronaviruses (CoV) of duck enteritis (DE-CoV) and porcine endemic diarrhea (PED-CoV) were isolated by bioassay on 3–5-day-old ducklings, followed by their adaptation to the permanent cell culture of the Vero line. Hemagglutinating encephalomyelitis virus (PHE-CoV) was isolated by bioassay on albino mice weighing 10–12 g, followed by an adaptation of the isolated virus to the permanent cell culture of the BHK-21 line. Virus-containing 10–30%

suspensions of the intestinal mucosa (DE-CoV and PED-CoV) or brain (PHE-CoV) were prepared on sodium phosphate buffer (pH 7.2–7.4). To get rid of the host fatty compounds they were shaken for up to 5 min with chloroform (final concentration 10–15%) at room temperature (RT, 18–24°C), followed by low-speed centrifugation (300–400 xg, 30 min, RT). Degreased tissue suspensions were passed through membrane filters in syringe cartridges with a pore diameter of 0.22 μm (Sartorius PVDF, Maharashtra, and analogs) before bioassay or cell culture inoculation.

Bioassay on ducklings was performed by oral injection to them (4–5 ducklings per isolate) of the above-mentioned suspensions the DE-CoV or PED-CoV in a dose of 1 ml. Suspensions of PHE-CoV virus were injected intraperitoneally in adult albino mice (2–3 mice per isolate) or to suckling mice (nests from 5–8 mice per isolate) — intracerebral: in doses of 0.5–0.7 ml and 0.05 ml, respectively. Control animals (mock) were injected with similar tissue extracts made according to the scheme described above from the same organs of healthy pigs or ducks from farms which free from coronavirus infections. Animals were kept under biosafety conditions that preclude their mix-infection and following the euthanasia requirements according to the Good Laboratory Practice principles (WHO, 2009). Clinical observations of infected and control animals were performed until the moment of their death or maximum the disease manifestation with each bioassay duration up to 10 days.

Adaptation of DE-CoV and PED-CoV isolates to Vero cell culture were performed using the standard procedure of trypsin treatment of inoculums at a final concentration of 1.5–4.0 mg/ml at an exposure of 45–60 min on 37°C (Hofmann and Wyler, 1988; Menachery et al., 2020).

Cell cultures of Vero and BHK-21 were grown and inoculated by the contact method using full monolayers of tissue culture in 25–50 ml flask or biological tubes as described elsewhere (Cutts et al., 2019).

As a control (mock) used tissue extracts from healthy pigs or ducks, which were manufactured according to the above-mentioned scheme. The cytopathic effect was recorded by microscopy and infectious activity of isolates was counted by the Reed-Mench method (Grimes, 2002).

Cell culture isolates of CoV were identified by hemaglutination (HA) and its inhibition (HI) tests using the following sera against coronaviruses:

(a) duck enteritis, manufactured by the former All-Union Research Institute of Veterinary Virology and Microbiology, Pokrov, Vladimir Region, Russia ($S^{DE\text{-}CoV\text{-}1989}$, VNIIVViM) and by the Kharkiv State Zooveterinary Academy, Kharkiv, Ukraine ($S^{DE\text{-}CoV\text{-}2002}$, KhSZVA);

(b) porcine endemic diarrhea, manufactured by the former VNIIVViM ($S^{PED\text{-}CoV\text{-}1999}$), as well as by the National Veterinary Research Institute, Puławy, Poland ($S^{PED\text{-}CoV\text{-}2005}$, PIWet) and by the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine ($S^{PED\text{-}CoV\text{-}2012}$, NSC 'IECVM');

(c) transmissible gastroenteritis of swine, produced by VNIIVViM ($S^{TGE\text{-}CoV\text{-}1993}$), as well as by PIWet ($S^{TGE\text{-}CoV\text{-}2003}$);

(d) bovine enteritis, produced by the former All-Union Institute of Experimental Veterinary Medicine, Moscow, Russia ($S^{BD\text{-}CoV\text{-}1990}$, VIEV) and NSC 'IECVM' ($S^{BD\text{-}CoV\text{-}2010}$).

HA-HI tests were performed in polystyrene plates with U-like wells using 0.8–1.0% suspension of mouse erythrocytes, viral hemagglutinins of DE-CoV, PED-CoV, and PHE-CoV, mentioned virus-specific sera — all according to the instructions for diagnosis 'Kit for diagnosis of bovine coronavirus by hemagglutination method'. IHA cross tests were conducted according to the titration scheme of the above sera with 8 HAU of each of the viruses, as we described previously (Semenikhin et al., 1994).

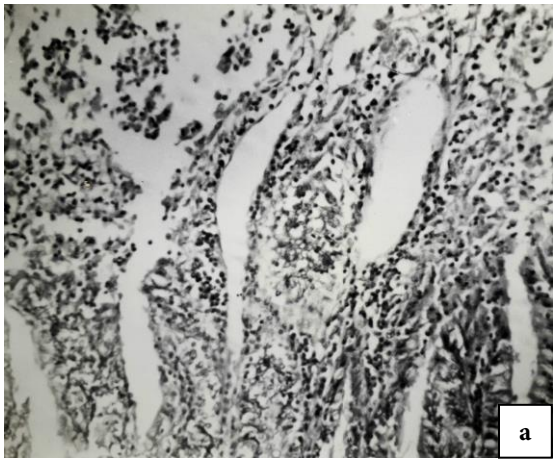
In addition, individual isolates of DE-CoV, PED-CoV, and PHE-CoV were identified by the classical negative stain method of electron microscopy (EM) using transmission electron microscope PEM-125K (OJSC SELMI, Ukraine), EM-grids with the phosphoric-tungsten acid films at the subject magnification of up to 60–80 thousand times (X).

Results. ***Trials on coronaviruses isolation.*** As we received the experience, the isolation of porcine and duck CoVs was the most successful when we began to use a bioassay with degreased tissue samples. In our trials, chloroform was the best choice for degreasing 10–30% tissue suspensions. Fig. 1 presents the illustrations of the results of the bioassay.

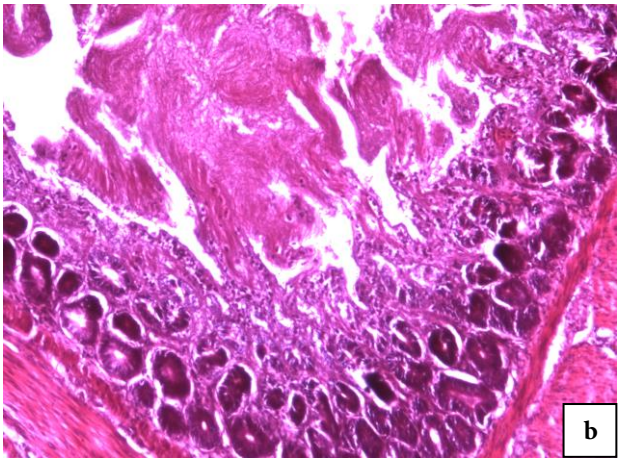
As shown in Fig. 1a–b, the manifestation of clinical signs in ducklings (drastic diarrhea, 'crawling by hocks': $n \approx 100$ for DE-CoV and $n = 18$ for PED-CoV) was escorted by the destruction of upper parts of the duodenum villi on 4–5th days for DE-CoV and PED-CoV isolates after administration *per os*. The PHE-CoV isolates which are known since 2008 ($n = 7$), evoke the porcine (Fig. 1f) and mouse (Fig. 1g) brain damage which is typical for encephalomyelitis. As a consequence of this brain damage, the diseased suckling piglets in enzootic holdings ($n = 3$) had a clinical sign of 'vomiting disease' and the disease in post-weaning piglets was manifested by 'wasting disease' (Fig. 1c) and convulsions/tremor signs in the terminal phase of the disease (Fig. 1d). The convulsions/tremor signs in the terminal phase of disease we observed also in mice (Fig. 1e) infected intraperitoneal ($n = 12$) and orally ($n = 4$).

The porcine and duck CoVs isolation in cell cultures was optimal when we began to use the specimens' trypsinization and application of trypsin (2–3 μg/ml) in maintenance cultural mediums at the first 3–5th passages of isolates.

Fig. 2 presents the illustrations of the PED-CoV (2 isolates) and PHE-CoV (1 isolate) cytopathology in Vero and BHK-21 cell lines, respectively.

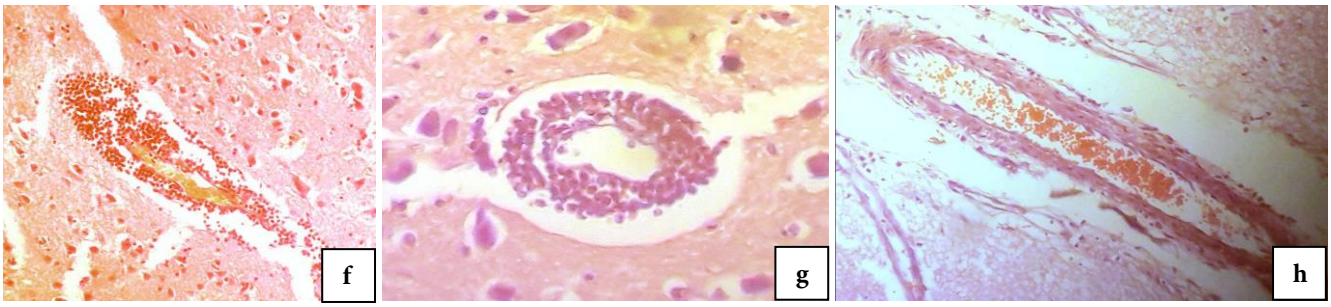


The DE-CoV isolate 'Taranivka-1991' (a): total destruction of upper parts of the ducklings' duodenum villi on the 4th day after oral administration (together with V. P. Knyazev, VNIIVViM, 1993)



The PED-CoV isolate 'Globino-2010' (b): total destruction of upper parts of the ducklings' duodenum villi on the 5th day after oral administration (together with M. M. Surkova, KhSZVA, 2015)

The PHE-CoV isolate 'Ryasne-2015': piglets under one month old with 'vomiting disease' and 'waste disease' in an age of 2.5–3.0 months (c); convulsions and tremor signs in terminal phase of disease in swine (d) and mouse (e) (together with O. V. Prokhoryatova, NSC 'IECVM', 2008)



The PHE-CoV isolate 'Lipczy-2008': the porcine (f) and mouse (g) brains with the typical vasculitis signs ('clotting' of leukocytes around blood vessels). In comparison with normal vessels (g, h) on the 7th day after intraperitoneal inoculation of mouse (together with P. O. Shutchenko, NSC 'IECVM', 2008)

Figure 1. Main results of bioassays on DE-CoV (a, b) and PHE-CoV (c–h) isolations. For more details, see the text

In Vero cells the 'Globino-2010' and 'Dnipro-2013' PED-CoV isolates reached titers 3.0–3.5 lg $TCID_{50}$/ml on the 3rd passage; the 'Sahnovsky-2013' PED-CoV isolate reached this titer only on the 7th passage. The first signs of the mentioned virus cytopathic effect were observed in 40–48 hrs p.i. (Fig. 2a–c). Full monolayer destruction by this PED-CoV dose occurred on the 5–7th day p.i.

The appearance of cytopathology in the case of PHE-CoV in BHK-21 cells monolayer occurred some faster than with PED-CoV in Vero cells.

Fig. 2d–e shows the typical cytopathic effect of the PHE-CoV isolate 'Lipczy-2008' already in 18 hrs p.i., while its infectious activity was 3.75 lg $TCID_{50}$/ml on the 2nd passage of 'mouse brain' virus.

Study of the coronavirus immunological relationships. Data on cross-reactions of CoVs hemagglutinins (8 HA-units) in HA-HI tests with the use of corresponding antiserums and mouse erythrocytes are summarized in Table 1.

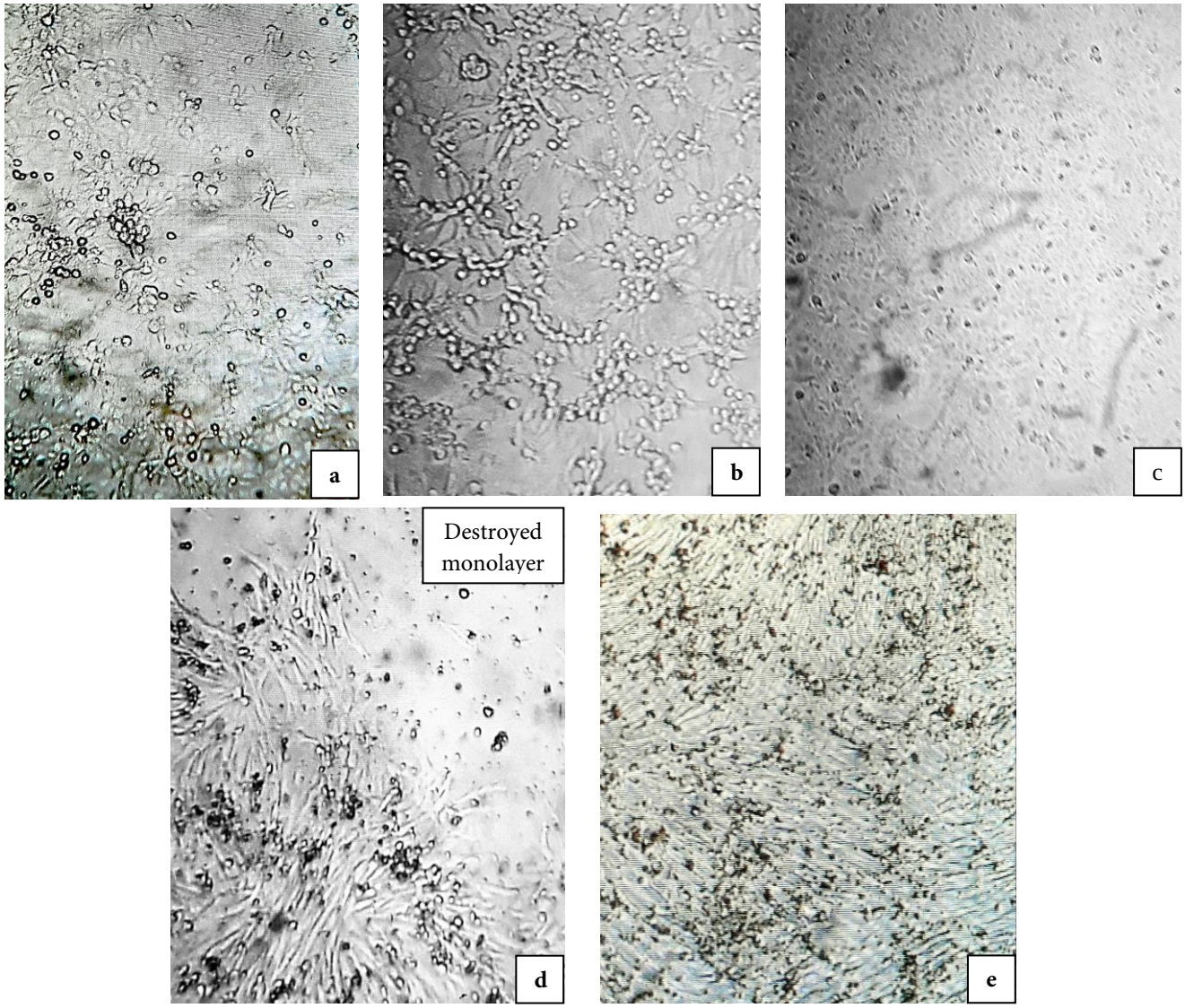


Figure 2. Cytophatology of the PED-CoV in Vero cells monolayer (a — isolate 'Globino-2010', the 3rd passage; b — isolate 'Dnipro-2013', the 2nd passage; c — mock) and PHE-CoV in BHK-21 cells monolayer (d — isolate 'Lipczy-2008', the 2nd passage; e — mock). For more details, see the text

Table 1 — Results of the HA-HI tests for study of CoVs immunological relationships

Antiserums against CoV*:	Haemagglutinins of CoV isolates (8 HA-units)					
	DE-CoV	PED-CoV			PHE-CoV	
	'Taranivka-1991'	'Globino-2010'	'Sahnovsky-2013'	'Dnipro-2013'	'Lipczy-2008'	'Ryasne-2015'
Reciprocal titers of duck antiserums against DE-CoV						
$S^{DE\text{-}CoV\text{-}1989}$	112.5 ± 29.12	28.1 ± 18.34	31.1 ± 10.23	57.3 ± 22.04	14.7 ± 1.54	n.d.
$S^{DE\text{-}CoV\text{-}2002}$	168.4 ± 27.91	42.1 ± 8.41	47.5 ± 17.77	64.4 ± 31.14	15.3 ± 0.33	n.d.
Reciprocal titers of porcine antiserums against PED-CoV						
$S^{PED\text{-}CoV\text{-}1999}$	22.3 ± 0.75	111.2 ± 9.12	100.9 ± 8.49	n.d.	n.d.	7.7 ± 3.91
$S^{PED\text{-}CoV\text{-}2005}$	29.5 ± 1.53	134.4 ± 7.73	88.7 ± 8.81	n.d.	n.d.	7.3 ± 4.86
$S^{PED\text{-}CoV\text{-}2012}$	23.2 ± 0.82	87.6 ± 9.71	79.5 ± 9.19	135.1 ± 7.55	n.d.	11.8 ± 1.29
Reciprocal titers of bovine antiserums against BovE-CoV						
$S^{BD\text{-}CoV\text{-}1990}$	97.2 ± 17.33**	n.d.	n.d.	n.d.	n.d.	n.d.
$S^{BD\text{-}CoV\text{-}2010}$	107.4 ± 31.12	76.3 ± 19.77	68.3 ± 19.88	53.8 ± 24.64	7.5 ± 2.05	n.d.
Reciprocal titer of mouse antiserum against PHE-CoV						
$S^{PHE\text{-}CoV}$	48.9 ± 2.13	12.4 ± 2.33	13.2 ± 3.24	13.4 ± 2.47	107.6 ± 2.88	122.8 ± 5.78

Notes: * — see descriptions in Materials and Methods; ** — together with N. L. Sokolova, 1994; n.d. — not done.

As we have no certified reference reagents for CoVs identification, our results should be considered preliminary. However, our data indicate a high probability of the definite immunological affinity of all the hemagglutinins learned PED-CoV and PHE-CoV isolates with hemagglutinins of DE-CoV. If we take the titers of homologous antiserum as 100%, then PED-CoV hemagglutinins are related to DE-CoV haemagglutinins (by bilateral affinity) by 24% vv.32% ($n = 8$, $P \le 0.01$), and to PHE-CoV hemagglutinins — by 35% vv. 42% ($n = 8$, $P \le 0.01$). At the same time, according to our results (together with N. L. Sokolova), hemagglutinins DE-CoV are related to bovine enteritis virus hemagglutinins by 73% ($n = 4$, $P \le 0.02$). bovine enteritis virus hemagglutinins are related to PED-CoV haemagglutinins by 54% ($n = 4$, $P \le 0.02$), but to PHE-CoV haemagglutinins — by 6% ($n = 5$, $P \le 0.01$) only.

Results of coronaviruses microscopy. Unexpected patterns of comparative CoVs morphology were revealed by the traditional negative contrast method of electron microscopy (Fig. 3). Fig. 3a–c presents the evidence of a high level of the DE-CoV polymorphism in environmental samples ($n = 25$): up to 70% of elongated and up to 15% pleomorphic and up to 15% rounded from all registered DE-CoV virions. At the same time, we can see the relatively homogenous morphology of PED-CoV virions (Fig. 3d–e) in its cultural samples ($n = 5$) — rounded particles up to 90% and pleomorphic virions (not shown — up to 10%). In the case of PHE-CoV (Fig. 3f–g) we can see the viral particles by 30% larger in size than DE-CoV virions; among them up to 90% of them were pleomorphic and up to 10% — rounded virions.

In other words, electron microscopy data show that DE-CoV samples contain virions of specific for DE-CoV morphology (elongated and even 'Ebola-like' particles, Fig. 3c) and with morphology that resemble and PED-CoV (rounded particles), and PHE-CoV morphology (pleomorphic particles).

Discussion and conclusions. Modern studies have shown chickens and Pekin ducks were not susceptible to SARS CoV-2 infection (Schlottau et al., 2020; Shi et al., 2020). In summer 2020 American scientific team had challenged the chickens, turkeys, ducks, quail, and geese with SARS-CoV-2 or MERS13 CoV. No disease was observed, no virus replication was detected, and antibodies were not detected in serum. Neither virus replicated in embryonated chicken eggs. Therefore poultry is unlikely to serve a role in the maintenance of either virus (Suarez et al., 2020). However, our data show that DE-CoV populations are very pleomorphic and contain some virions which morphologically are indistinguishable from viruses PED-CoV and PHE-CoV (Fig. 3) and are closely related to them by immunology (Table 1). Therefore we can suggest a real risk of ducks participating in the emergence of at least alpha-coronavirus infections in pigs. This hypothesis is based also on our preliminary investigations.

Brief, duck coronaviral enteritis was diagnosed in USSR from 1987 to 1995 in the frame of special observations in 9 duck industrial state holdings in Russia (North Caucasus, Southern Volga, and Central Regions, West Siberia), and also in Ukraine and Belarus (Lagutkin et al., 1994).

In 1987, the 'Ivano-Frankivsk virus' (isolate 'IF-87') was isolated from samples of organs of dead ducklings of 5–30 days of age from the 'Gorodenkivska' poultry farm (Ukraine) in the former All-Union Research Institute of Veterinary Virology and Microbiology, Pokrov, Vladimir Region, Russia (Knyazev et al., 2003).

According to electron microscopy data, viral particles of the pathogen were polymorphic: spherical, elongated, and curved; the sizes of spherical virions ranged from 80 to 200 nm, and elongated and curved — from 70 to 300 nm. On the surface of the particle envelope, clavate peplomers with a length of 7 to 12 nm were found, forming a crown. A thickened core was found in the center of all viral particles (Buzun et al., 1995a).

The duck disease by clinic, epizootology, and pathology resembled the porcine transmissive gastroenteritis (Buzun et al., 1995b, 1995c).

According to state statistics in the 1980s, industrial farms, where the breeding stock was staffed with Peking ducks imported from China, gradually lost their profitability due to the growing year by year lethality of ducklings. Particularly catastrophic consequences in state farms were caused by the mass placement of 'Medeo' and 'Temp' crosses, which were bred in Kazakhstan and Belarus, respectively, based on breeding ducks of Peking breed from China (duckling lethality to 70–90%). Subsequent studies have shown the affinity of hemagglutinins of viruses DE-CoV and BE-CoV (Semenikhin et al., 1996).

Much more, the nucleocapsids of viruses DE-CoV and TGE-CoV were revealed as affinity in immunoblotting; under experimental conditions was proved the prophylactic efficacy of intranasal application to 1–2-day-old ducklings ($n = 1{,}300$) of the virus vaccine against TGES from the strain 'VGNKI' in doses of 400–800 $TCID_{50}$ (once, intranasal the 1.5–2.0 ml) (Musukaeva, 1997).

This approach was not introduced in post-soviet practice — only stamping out with the replacement of duck herds. But we can not vouch that TGE-vaccination in duck industry was not conducted off outrange of the former USSR and it not had as consequence the appearance the new porcine CoV — i.e. PED-CoV. In this context, against the background of the COVID pandemic, we expect an increase in the incidence of pigs for beta-coronavirus infections — in particular PHE-CoV.

Acknowledgments. We are grateful to all colleagues who helped us to conduct these investigations and to summarize the results, and will not lose the hope to continue the development of this topic.

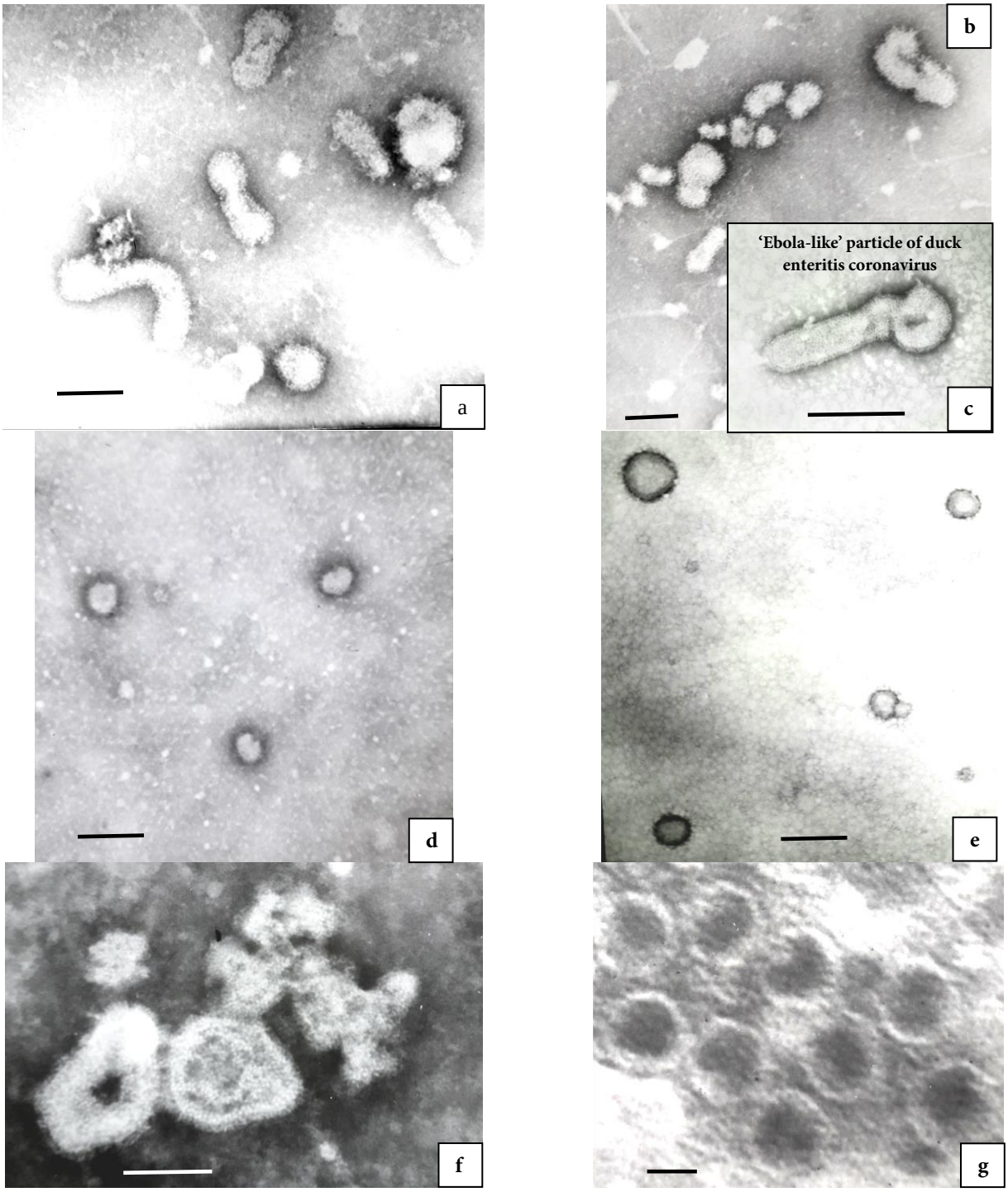


Figure 3. Negative-stain electron microscopy image of: a–c — DE-CoV isolate 'Taranivka-1991', environmental sample (fatty cap of slurry collector, together with M. Malahova, 1993); d–e — PED-CoV isolate 'Globino-2010': samples of porcine intestine (d) and Vero cells (e, the 3rd passage of PED-CoV); f–g — PHE-CoV isolate 'Ryasne-2015': samples of mouse brain (f) and BHK-21 cells (g, the 2nd passage of PHE-CoV). The magnification bars in the pictures represent 100 nm in length. For more details, see the text

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EPIDEMIC SITUATION OF AVIAN INFLUENZA IN UKRAINE AND IN THE WORLD DURING 2019–2020

Sapachova M. A., Sushko M. I., Mandyhra S. S., Movchun O. M., Skorokhod A. O., Posna O. V., Terletska K. M., Zahrebelnyi V. O., Mezhenskyi A. O.

State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Kyiv, Ukraine, e-mail: mandygra@ukr.net

Summary. The aim of the work was to analyze current epidemic situation on highly pathogenic avian influenza worldwide and in Ukraine during 2019–2020. The research was conducted in the framework of state control of infectious animal diseases. The results of PCR tests obtained in the State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise and in regional state laboratories, data of OIE website (http://www.oie.int) in 2019 and 2020 were analyzed and summarized. During 2019–2020 outbreaks of highly pathogenic avian influenza were detected in 53 countries of the World: in Europe — 22, in Asia — 21, in Africa — 7, in America — 2, and in Australia — 1. In 2019 there was not registered any case of the highly pathogenic avian influenza in Ukraine. In 2020 nine outbreaks of avian influenza were registered in commercial poultry farms (n = 2) and backyards (n = 7) of Vinnytsia, Mykolaiv, Kyiv, and Kherson regions of Ukraine. It was confirmed that all characterized viruses were closely related and belonged to the highly pathogenic avian influenza virus H5N8 clade 2.3.4.4b identical to the avian influenza viruses currently circulating in Europe and Asia. Wild birds were identified as the main modes of HPAIV spread

Keywords: highly pathogenic avian influenza, H5N8, outbreaks

Introduction. Avian influenza (AI) is an infectious and contagious viral disease of birds. The etiological agent is a negative-sense, single-strand RNA virus with a segmented genome that belongs to the Orthomyxoviridae family *Influenzavirus A* genera. Type A influenza virus can affect wild birds as well as food producing birds (chickens, turkeys, quails, guinea fowls, etc.) and pet birds, some subtypes of virus cause high mortality rates. The virus has also been isolated from mammalian species including humans. Based on the antigenic structure of the surface glycoproteins type A influenza viruses have been classified into 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes that replicates in birds in different combinations (Alexander, 2007; Cox et al., 2000). Recently, two additional HA and NA subtypes (H17N10 and H18N11) subtypes have been detected in bats (Tong et al., 2012, 2013). Theoretically, thousands of different combinations of HA and NA are possible.

Avian influenza viruses (AIVs) commonly detected in the wild birds in most cases are avirulent or cause only mild clinical signs (reduced body weight) (Feare, 2010; The Global Consortium for H5N8 and Related Influenza Viruses, 2016), and are referred to low pathogenic avian influenza virus (LPAIV). However, LPAIV subtypes have the capacity to mutate and become high pathogenic avian influenza virus (HPAIV) — a systemic disorder with an acute clinical course and high mortality rate (Alexander, 2007). It is supposed that the transformation occurs during transmission from wild birds and adaptation of LPAIV to poultry (Feare, 2010; The Global Consortium for H5N8 and Related Influenza Viruses, 2016). From the 16 HA subtypes of AIV detected in wild birds, only H5 and H7 subtypes (regardless of the N subtype) have the potential to mutate. According to the World Organisation for Animal Health (OIE), AIV is defined as 'an any influenza A virus with high pathogenicity (HPAI) and H5 and H7 subtypes with low pathogenicity (H5/H7 LPAI)'. OIE requires notification for all H7 and H5 subtypes, regardless of their pathogenicity (OIE).

The economic impact of AIV on poultry is severe, since not only the decreasing of egg production and a high mortality (may reach 100%) in birds are observed, but also a significant restriction in trade. Another subject of concerning is the ability of AIV to genetic reassortments that can lead to development of advanced human strains (Feare, 2010; Gaidet et al., 2010; King et al., 2020).

AIVs are spread globally thanks to reservoirs such as birds. Due to passage through the territory of Ukraine of a large number of transcontinental migration routes of wild migratory birds (pass from North Asia and Europe to the Mediterranean, Africa, and Southwest Asia and cross from the Baltic and Caspian seas to the Black and Mediterranean seas, and from Western Siberia and Kazakhstan to Western Europe and North Africa), sharing common border with countries where cases of AI were registered and trade relations with these countries, there is a high risk of introduction of AIV into the territory of Ukraine (Muzyka et al., 2012, 2017; Sapachova et al., 2019).

The necessity of epidemiological surveillance on AI determined by the risk of introduction of new virus strains into the country and penetration of the virus into commercial poultry farms and backyards, the emergence of epidemics that can lead to great economic losses.

The aim of the study was to analyze epidemic situation on HPAI in the World and in Ukraine during 2019–2020.

Materials and methods. The research was conducted in the framework of state control of infectious animal diseases. The results of PCR tests obtained in the State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise and in regional state laboratories in 2019 and 2020 were analyzed and summarized. Data of OIE website (http://www.oie.int) and materials of scientific publications were used.

Samples. During 2019–2020, 7,934 samples of internal organs (intestines, lungs, heart, and trachea), cloacal and tracheal swabs from poultry were tested. In addition, 5,659 wild birds (mainly comprising duck, swans, and pigeons) were examined in the same period. A standard set of organ samples, oropharyngeal and cloacal swabs, poultry manure were collected from dead or shot wild birds.

Virus detection and subtyping. Cloacal and tracheal swabs were immersed in 1 ml of phosphate buffered saline (PBS), shaken and centrifuged for 10 min at 3,000 g. From organ samples 10% suspensions were prepared, centrifuged for 10 min at 3,000 g. Total RNA was extracted from 0.2 mL of supernatant using a IndiSpin Pathogen Kit (Indical Bioscience, Germany) and reagents for column isolation of RNA/DNA 'ArtRNA MiniSpin' (ArtBioTech, Belarus) according to the manufacturer's protocol. The detection of AIV RNA (detection of the M gene) was performed using a VetMAX™-Gold AIV Detection Kit (Avian Influenza Virus RNA Test Kit) (Thermo Fisher Scientific). In the case of positive samples, the subtyping hemagglutinins H5 and H7 were carried out by Adiavet AIV H5-H7 Real Time (Adiagène SARL, France). RT-PCR for the N8 gene were carried out using AgPath-ID™ One-Step RT-PCR Reagents (Ambion-Applied Biosystems) with N8 RT-PCR primers and probe, described by Hoffmann et al. (2016).

Virus sequencing. The pathogenicity of AIV were determine according to a SOP 'One Step PCR for detection of H5 and H7 avian influenza virus and cleavage site sequencing' used by the OIE/FAO international reference laboratory for AI at the Animal and Plant Health Agency (Weybridge, UK).

Samples containing AI virus type A subtype H5N8 were submitted to OIE reference laboratory (Animal and Plant Health Agency, Weybridge, UK) for AIV pathogenicity determination and full genome sequencing. The RNA nucleotide sequences were compared to nucleotide sequences of RNA viruses circulated in Europe and Asia, nucleotide sequences of RNA viruses were obtained from NCBI and GISAID EpiFlu databases. Sequence comparisons were performed with the BLAST.

Results and discussion. During 2019–2020 outbreaks of highly pathogenic avian influenza were registered in 53 countries of the World: in Europe — 22, Asia — 21, Africa — 7, America — 2, and in Australia — 1 (Table 1).

According to data shown in Table 1, the most diversity of various H5 subtypes of HPAIV circulate in Asia (H5N1, H5N2, H5N5, H5N6, and H5N8). In Africa H5N1, H5N2, H5N6, and H5N8 were detected. In most European countries circulating of H5N8 of HPAIV were observed, H5N6 was detected only in Denmark and Ireland, H5N1 in Italy, United Kingdom, and the Netherlands. H7 subtypes of HPAIV were detected in America (H7N3 in Mexico, USA) and Oceania (H7N7 in Australia).

Table 1 — Circulation of HPAIV (types H5 and H7) in the World in 2019–2020

AIV type	Country
H5	
H5N1	Bhutan, China, India, Laos, Nepal, Nigeria, Togo, Vietnam, Italy, United Kingdom, The Netherlands
H5N2	Chinese Taipei, Egypt
H5N5	Belgium, Chinese Taipei, Denmark, Germany, United Kingdom, Sweden, Taiwan, The Netherlands
H5N6	Cambodia, China, Denmark, Ireland, Nigeria, Philippines, Vietnam
H5N8	Belgium, Bulgaria, Czech Republic, Congo, Denmark, Croatia, France, Germany, Hungary, Iran, Iraq, Italy, Ireland, Israel, Japan, Kazakhstan, Kuwait, Korea, Namibia, Nigeria, Norway, Pakistan, Poland, Romania, Russia, Ukraine, United Kingdom, Saudi Arabia, Slovakia, Slovenia, South Africa, South Korea, Sweden, Spain, The Netherlands
H7	
H7N3	Mexico, United States of America
H7N7	Australia

The analysis of an epidemiological situation of AI in Ukraine during 2019–2020 was carried out (Tables 2–3). In 2019–2020, 13,593 samples of pathological material and swabs were tested by RT-qPCR, which includes 5,659 samples from wild birds and 7,934 — from domestic. Samples of internal organs (intestines, lungs, heart and trachea), cloacal and tracheal swabs, as well as poultry manure were taken for the testing.

Table 2 — The results of AIV type A detection in pathological material from birds

Year	Object of study					
	Poultry			Wild birds		
	Tested samples	Positive	%	Tested samples	Positive	%
2019	111	0	0	2,367	0	0
2020	7,823	149	1.90	3,292	4	0.12
Total	7,934	149	1.88	5,659	4	0.07

In 2019 positive samples on AIV type A were not detected. In 2020, 153 positive samples on AI type A (149 samples from poultry and 4 — from wild birds)

were detected. All AI positive samples were typed on H5 and H7 subtypes.

According to the data in Table 3, presence of AIV subtype H5 were confirmed only in 54 samples of pathological material and in 30 samples of cloacal and tracheal swabs of poultry of Vinnytsia, Kherson, Kyiv and Mykolaiv regions. In sample of pathological material of poultry of Ternopil, Odesa, Kharkiv regions and samples of pathological material of wild bird of Odesa and Kherson regions H5 and H7 subtypes of AIV type A were not detected.

In general 9 outbreaks of AI there were registered in Vinnytsia (1), Mykolaiv (5), Kyiv (2) and Kherson (1) regions (Table 4).

Table 3 — The results of AIV type A subtyping on hemagglutinins H5 and H7

No	Region	Type of sample	Number of samples	Hemagglutinin subtype	
				H5	H7
1	Vinnytsia	pathological material of poultry	36	25	0
		swabs (cloaca and trachea) of poultry	30	30	0
2	Ternopil	pathological material of poultry	1	0	0
3	Odesa	pathological material of wild birds	2		
		pathological material of poultry	19	0	0
4	Kherson	pathological material of wild birds	2	0	0
		pathological material of poultry	5	5	0
5	Kyiv	pathological material of poultry	6	6	0
		poultry manure	1	0	0
6	Mykolaiv	pathological material of poultry	35	18	0
7	Kharkiv	pathological material of poultry	16	0	0
		Total	153	84	0

Table 4 — Outbreaks of AI in Ukraine in 2020

Date	Outbreak location	Susceptible	Cases	Deaths	Killed	Morbidity rate, %	Mortality rate,%	Fatality rate,%
18.01.2020	Buhakiv, Nemyriv District, Vinnytsia Region (farm)	116,544	7,856	7,856	108,688	6.74	6.74	100
02.12.2020	Kandybyne, Nova Odesa District, Mykolaiv Region (backyard)	10	10	10	0	100	100	100
08.12.2020	Novomatviivske, Nova Odesa District, Mykolaiv Region (village)	115	3	0	20	2.61	0	0
23.12.2020	Yaselka, Ochakiv District, Mykolaiv Region (backyard)	21	3	3	18	14.29	14.29	100
23.12.2020	Leonivka, Ivankiv District, Kyiv Region (village)	463	463	463	0	100	100	100
26.12.2020	Borodianka, Borodianka District, Kyiv Region (backyard)	1	1	1	0	100	100	100
28.12.2020	Trykhaty, Mykolaiv District, Mykolaiv Region (backyard)	16	1	1	15	6,25	6.25	100
28.12.2020	Ivanivka, Arbuzynka District, Mykolaiv Region (farm)	98,264	587	587	15	0.60	0.60	100
28.12.2020	Zaozerne, Kakhovka District, Kherson Region (backyard)	43	26	26	17	60.47	60.47	100

The first outbreak of AI was registered in an industrial poultry farm with a total number of layers over 98,000 in Vinnytsia Region (Buhakiv Village of Nemyriv District) on 18 January 2020. The outbreak was detected in the course of passive surveillance at an establishment for laying hens. It was the first outbreak of AI caused by HPAI H5N8 virus in our country over the last 3 years. The presence of HPAIV type A subtype H5N8 in the poultry samples were confirmed by OIE reference laboratory in Animal and Plant Health Agency (Weybridge, UK). Whole genome sequence analysis was shown high identity with European H5N8 viruses (i.e. Poland and Slovakia 2020) clade 2.3.4.4b. The genotype of the virus is the same as those viruses that have been detected in domestic and wild birds in Europe since December 2019. They are reassortants of LPAI viruses from Eurasia and HPAI A (H5N8) viruses from Africa. In particular, PB1 and NP genes derive from Eurasian

wild bird strains and the remaining genes are most similar to viruses circulated in West Africa in 2019. It is likely that this reassortment occurred in wild migratory birds in Asia during the summer and then spread to Eastern Europe with the autumn migration of birds.

The first outbreak was quickly eradicated. Correct and quick reaction on this first outbreak that was slaughtering of all susceptible poultries in the farm (108,688 birds) and undertaken veterinary and sanitary measures helped to stop spreading of the AIV.

Another eight outbreaks of HPAIV were registered during December 2020.

AI outbreaks were registered in five districts of the Mykolaiv region: two outbreaks in backyards in Nova Odesa District (in Kandybyne Village 02.12.2020 and in Novomatviivske Village 08.12.2020); single outbreaks in backyards in Ochakiv (Yaselka Village 23.12.2020) and Mykolaiv districts (Trykhaty Village, 28.12.2020); and on an industrial poultry farm in Arbuzynka Ditrict (Ivanivka Village 28.12.2020).

In Kyiv Region two AI outbreaks were registered. First outbreak of AIV type A subtype H5N8 were registered 23.12.2020 in poultry (23 turkeys and 440 chickens) founded on a roadside in a distance 1 km from Leonivka Village Ivankiv District. Second outbreak of AI was recorded 26.12.2020 in a backyard in Borodianka Village Borodianka District during an epidemiological investigation of the AI outbreak in Ivankiv District.

28.12.2020 AI outbreak was registered in poultry in a backyard in Zaozerne Village Kakhovka District Kherson Region.

The source of infection has not been found yet for any of the registered outbreaks of AI. Wild birds are the most likely source of AIV in our country, even considering the fact that in farms the poultry did not have outdoor access. Indirect contact with infected wild birds thought polluted environment by infected birds can lead to AIV spreading. Analysis of migration routes of wild birds indicates that Mykolaiv, Odessa, and Kherson regions are located at the crossing of wild bird migratory routes, so the risk of introduction of AIV by wild migratory birds is high.

The higher number of outbreaks observed in poultry and it absence in wild birds could be explained by an ineffective passive surveillance of wild birds, by a different host response to virus infection between domestic and wild birds, or by herd immunity of wild birds due to past seasons' exposure to HPAI A (H5).

Conclusions. During 2020, nine outbreaks of AI were detected in two commercial poultry farms and in seven backyards of Vinnytsia (1), Mykolaiv (5), Kyiv (2), and Kherson (1) regions in Ukraine. It was confirmed that all isolated viruses were closely related and belonged to the HPAIV H5N8 clade 2.3.4.4b identical to the AIV that are currently circulating in Europe and Asia. Wild birds and human activity were identified as the main modes of HPAIV spread. Further epidemiological studies should be directed to identifying possible risk factors and understanding routes of AIV spreading. Systematic surveillance and prevention epidemiological measures need to be continuing.

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Part 2. Biotechnology

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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE DETECTION OF *BRUCELLA OVIS* DNA IN CLINICAL SAMPLES

Marchenko N. V., Lymanska O. Yu., Gerilovych A. P., Bolotin V. I.

National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: marcenkonata@gmail.com

Summary. The etiological agent of infectious ovine epididymitis is *Brucella ovis* and for its direct indication in clinical samples several PCR protocols are proposed. This study describes a design and selection of the oligonucleotides for real-time PCR targeting conservative BOV_A0504 gene. The specificity of a real-time PCR was validated using 25 *B. ovis* field isolates and 14 microorganisms of closely related species. The detection limit of *B. ovis* in bacterial culture was determined as 3.5×10^1 CFU/mL with Ct value of 37.8. There are no detectable fluorescence signals in the clinical samples from intact animals, whereas bacteriologically confirmed material such as urine and testicle tissue samples were positive. It confirms that the assay is highly specific for detection of *B. ovis* DNA. Thus, the proposed real-time PCR assay enables fast detection and quantification of *B. ovis* in clinical material, which can be used as additional test for estimation of the health status of a sheep herd

Keywords: specific primers, infectious ovine epididymitis, sheep

Introduction. *Brucella* (*B.*) *ovis* is associated with the infectious ovine epididymitis (IOE) that is one of the most important infectious diseases in sheep causing significant economic losses worldwide (OIE, 2018).

This infection is characterized by epididymitis and orchitis in rams, abortion in ewes as well as birth of weak lambs.

The diagnosis is based on the gold standard methods such as serology (CFT, ELISA and AGID) and pathogen isolation (Alton et al., 1988).

As an additional and express test several molecular techniques were elaborated, including conventional PCR (Bricker, 2002; Manterola et al., 2003; Xavier et al., 2010), nested PCR (Costa et al., 2013), and multiplex PCR (Saunders et al., 2007; López-Goñi et al., 2008; Moustacas et al., 2013). These assays are highly specific and faster than serological and bacteriological methods. Moreover PCR could help to confirm *B. ovis* circulating in some clinical cases or during bacterial culture characterization. Real-time PCR is an effective molecular tool allowing quantification of target DNA without electrophoresis that is less time-consuming.

For the differentiation among *Brucella* spp. species a real-time PCR assay have been developed (Hinić et al., 2008) as well as a protocol for simultaneous *B. ovis* and *H. somni* indication in urine and sperm samples (Moustacas et al., 2015), but there is a lack of *B. ovis*-specific protocol.

Thus, **the aim of this study** was to design specific primers and probe as well as develop real-time PCR protocol for *B. ovis* DNA detection in clinical samples.

Materials and methods. Oligonucleotide sequences for the real-time PCR primers and probe were designed using AmplifX v. 2.0.7 (https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr) and BioEdit v. 7.0.0 (Rozen and Skaletsky, 2000) based on sequence data available from GenBank (https://www.ncbi.nlm.nih.gov/genbank). These sequences were compared using the basic local alignment search tool BLAST (https://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Primers and the FAM-labeled probe were commercially synthetized.

To assess analytical specificity of real-time PCR a panel of DNA samples was prepared, which included 25 field isolates of *B. ovis*, reference strain *B. ovis* 63/290, that was kindly provided by Dr. Claire Ponsart (ANSES, France), other species of the genus *Brucella*, *Yersinia enterocolitica*, *Campylobacter fetus* subsp. *fetus*, *Corynebacterium pseudotuberculosis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. Enteritidis (all strains were obtained from the National Collection of Microorganisms of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine).

For DNA extraction 3–5 colonies of each microorganism were transferred into 200 µL of sterile saline buffer and boiled at 95°C for 10 min. The cell lysate was centrifuged at 17,380 g for 1 min. The 5 µL of supernatant was used for amplification.

Clinical materials (urine, vaginal swabs, testicles and lymph nodes) were sampled from infected and intact animals in two farms in Kharkiv Region. DNA extraction was done using innuPREP DNA/RNA Mini Kit (Analytic, Jena, Germany) following the manufacturer's instruction.

The DNA concentration was estimated using DeNovix DS spectrophotometer and converted to copy

number based on an average genome size of 3.28 Mb estimated from completed *B. ovis* ATCC 25840 genome available in GenBank (accession number CP000708 and CP000709). The standard DNA copies were prepared based on the size of the genome. Sensitivity of the method was proved by testing of *B. ovis* DNA in dilution from 10^7 to 10^1 to determine the detection limit of bacterial copy number. The coefficient of determination (R^2) and the slope value (s) of the regression curve were calculated.

PCR was performed in a 25 μL reaction volume using 2xABsolute Blue QPCR Mix, low ROX in plates MicroAmp® and 7500 Real-time PCRSystem (Applied Biosystems, USA) due to the optimal reaction conditions such as incubation times and temperatures, concentrations of the primers and dNTPs.

The detection of a fluorescent signal was done by the FAM channel (492–516 nm) and data was registered during the extension step. The results were interpreted by the presence (or absence) of the intersection of the fluorescence curve with the threshold level (Ct) set at the appropriate level. The sample was considered positive if for this sample the Ct value ≤ 38 on the FAM channel; if the value of Ct was absent (the fluorescence curve does not cross the threshold line) or greater than 38 the sample was considered negative or questionable respectively. Agreement between culture and real-time PCR was assessed by Kappa test.

Results and discussion. To develop a PCR assay for the indication of *B. ovis* genetic material, available complete genomes and different genes (IS711, 16S and 23S rRNA, Ykwd, HSP70, HSP40, CSP, Omp2, Omp31, ABC transporter and others) of *B. ovis* strains were analyzed and specific primers and probe were selected.

To select promising DNA targets, the publication regarding the comparison of complete genomes of the pathogens was analyzed (Rajashekara et al., 2004).

For the differentiation of *B. ovis* loci which are partially or completely absent at representatives of this kind can be used, namely: BMEI0899-0907, BMEI0993-1012, BMEII0129, BMEII0185-0226, BMEII0405, BMEII0708, BMEII0811-0815, BMEII0875-0878. The genes BMEI0994 and BMEII0812 were promising for the further study.

The first locus is located in the region of BMEI0993-1012, the genes of which are involved in the genetic markers of the Bruce-Ladder system (BMEI0998-0997), as *B. ovis* characterization (López-Goñi et al., 2008).

The second locus is specific according to the results of analysis *in silico*. Therefore, in order to develop a test system for the indication of *B. ovis* genetic material by real-time PCR, we additionally searched for conservative regions of pathogen's genes to construct specific primers and probes. To do this, we studied openly published complete genomes and genes using GenBank nucleic acid sequence databases and bioinformatics using BioEdit v. 7.0.0 software. In general, 71 genes of different strains of *B. ovis* were analyzed.

It was found that the considered sequences are not suitable for the primers construction, because the divergence within these genes of different *Brucella* species is only 3%. Therefore, the complete genome of the reference strain *B. ovis* ATCC 25840 was further analyzed.

Based on the results of this work, genes encoding hypothetical conserved proteins BOV_0019, BOV_0245, BOV_0650, BOV_0651, BOV_0950, BOV_1227, BOV_12284, BOVA were selected. Among the selected sequences, the genes BOV_A0504 and BOV_A0509 were promising for the construction of primers, and the last one is close to the gene BPI_II597 of *B. pinnipedialis* B2/94.

For the further primers construction for PCR detection of *B. ovis* DNA, the BOV_A0504 gene was selected. Using the program AmpliFx by iterations determined the sequences of primers based on their thermodynamic characteristics — melting point and enthalpy (Table 1).

Table 1 — Selection of the primer sequences using the AmpliFx soft

Name	Sequence	Start position	Direction	Length, number of nucleotides	Temperature, °C
Primer Set 1: 100 bp					
query_L1	AGAAGCTGTCGCCATAAGGT	1470	Forward	20	56.12
query_R1	TTGTCTGCAGTTCTGTTGGC	1550	Reverse	20	56.06
query_P1	CCCGAACGCGGTTCCTCCGC	1511	Forward	20	65.55
Primer Set 2: 100 bp					
query_L2	ACGAGTCTATCGTCGGACAC	1322	Forward	20	56.13
query_R2	CAAAGGCTATTCACCGCACA	1402	Reverse	20	55.93
query_P2	CGCAAGACCAAGCACCGGCT	1354	Forward	20	63.11
Primer Set 3: 105 bp					
query_L3	ATCAATCGGCTGCTTTAGCG	1084	Forward	20	56.18
query_R3	ATTAAGGGCGCCGAGGAATA	1169	Reverse	20	55.96
query_P3	CGGAAATTTCCTTAGCCTTACCCTGCG	1140	Forward	27	62.07

Thus, the third set of the sequences was selected, which flanks a site of 105 bp. These sequences were also analyzed using the BLAST algorithm to confirm their specificity *in silico*. Synthesized primers were used for *in vitro* amplification and the reaction was optimized regarding cycling parameters: 94°C for 2 min followed by 40 cycles of 94°C, 15 s and 60°C, 30 s.

To obtain a standard curve serial 10-fold dilutions of *B. ovis* DNA and corresponding Ct values were used (Fig. 1).

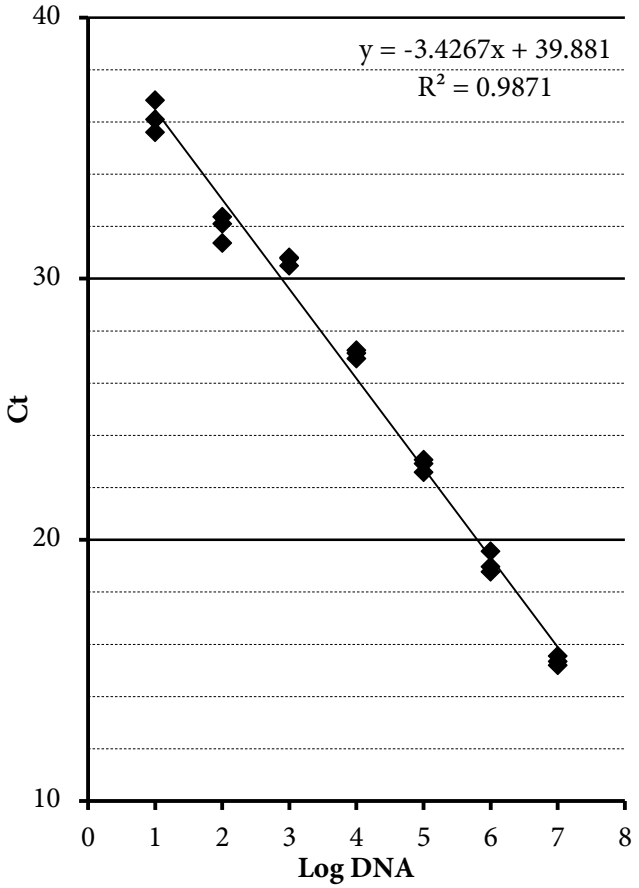

Figure 1. Standard curve showing the relationship between Ct values and standard dilution series of DNA *B. ovis* 65/65939 (Slope value = –3.427; R^2 = 0.987; Amplification factor = 1.96; qPCR Efficiency = 95.8%)

The obtained data showed that linearity was maintained (slope = –3.427 and coefficient of determination $R^2 > 0.98$). There is linear correlation between template concentrations ranged from 10^1 to 10^7 copies of DNA. The detection limit of *B. ovis* in bacterial culture was determined as 3.5×10^1 CFU/mL with Ct value of 37.8.

To assess an analytical specificity of the test a panel of DNA samples, isolated from 25 *B. ovis* strains and other bacteria, was studied. The results are showing in Table 2.

None of the bacterial species except *B. ovis* gave positive Ct value.

For the estimation of the diagnostic specificity a panel of DNA extracted from different clinical samples was tested (Table 3).

Table 2 — The specificity of the *B. ovis* real-time PCR assay by testing of DNA samples obtained from different bacteria

No	Strain	Ct, Fam (Mean ± SD; n = 3)	Result
1	*B. ovis* 67/Б	21.6 ± 0.34	+
2	*B. ovis* 76/982	26.1 ± 0.21	+
3	*B. ovis* 83/7315	24.3 ± 0.65	+
4	*B. ovis* 103/33479	25.1 ± 0.58	+
5	*B. ovis* 156/7808	22.1 ± 0.22	+
6	*B. ovis* 159/8406	22.0 ± 0.93	+
7	*B. ovis* 162/08337	23.0 ± 0.32	+
8	*B. ovis* 166/13575	24.0 ± 0.52	+
9	*B. ovis* 168/1807	21.7 ± 0.18	+
10	*B. ovis* 175/1257	24.3 ± 0.8	+
11	*B. ovis* 179/00441	23.6 ± 0.24	+
12	*B. ovis* 182/Taison	23.1 ± 0.15	+
13	*B. ovis* 183/13206	21.3 ± 0.41	+
14	*B. ovis* 186/21116	23.8 ± 0.24	+
15	*B. ovis* 64/64	26.2 ± 0.84	+
16	*B. ovis* 74/139	20.0 ± 0.03	+
17	*B. ovis* 154/8206	19.0 ± 0.08	+
18	*B. ovis* 155/8162	18.7 ± 0.15	+
19	*B. ovis* 158/4151	22.4 ± 0.2	+
20	*B. ovis* 178/00440	21.0 ± 0.03	+
21	*B. ovis* 181/6967	22.0 ± 0.49	+
22	*B. ovis* 184/03785	23.4 ± 0.03	+
23	*B. ovis* 185/03377	20.4 ± 0.7	+
24	*B. ovis* -/644	20.8 ± 0.91	+
25	*B. ovis* 65/c	19.4 ± 0.59	+
26	*B. abortus* 544	0.00	–
27	*B. abortus* 88/7-26	0.00	–
28	*B. suis* 58/1330	0.00	–
29	*B. melitensis* Rev-1	0.00	–
30	*B. canis* M20	0.00	–
31	*Y. enterocolitica* O:9	0.00	–
32	*Y. enterocolitica* O:3	0.00	–
33	*C. fetus* subsp. *fetus*	0.00	–
34	*L. monocytogenes*	0.00	–
35	*S. enterica* subsp. Enteritidis	0.00	–
36	*E. coli*	0.00	–
37	*S. aureus*	0.00	–
38	*C. pseudotuberculosis*	0.00	–
39	*P. aeruginosa*	0.00	–

Table 3 — Diagnostic specificity of the *B. ovis* real-time PCR assay by testing of DNA samples obtained from different clinical material

Sample	Ct, Fam (Mean ± SD)
Urine from infected rams (n = 5)	32.4 ± 0.82
Testicle tissues and lymph nodes from infected rams (n = 6)	30.64 ± 1.19
Samples from intact sheep (n = 42)	0.00

There are no detectable fluorescence signals in the clinical samples from intact animals, whereas bacteriologically confirmed material such as urine and testicle tissue samples were positive. It confirms that the assay is highly specific for detection of *B. ovis* DNA. Additionally, two samples of the testicle tissues were positive in real-time PCR, but the pathogen was not isolated (Kappa = 0.896).

High specificity and short period of testing are the most important advantages of PCR. It is an effective additional tool for routine laboratory diagnostic for the *B. ovis* detection in clinical samples. However, due to the OIE recommendations indirect diagnosis based on serological tests is preferred for herd surveillance (OIE, 2018).

The real-time PCR is recommended as additional method for early diagnostics and prevention of IOE during herd completing and could be widely used in the practice of state veterinary laboratories. Real-time PCR for detection of *B. ovis* DNA in clinical samples is a fast and reliable alternative for culturing.

Conclusion. A rapid, specific and sensitive quantitative PCR assay for the detection of *B. ovis* was developed. Its application makes possible to confirm the diagnosis in short time, to improve the efficiency of rehabilitation of sheep in problematic positive herds. PCR analysis is recommended as additional test for early diagnosis and prevention of IOE during herd recruitment and can be widely used in the practice of state veterinary laboratories.

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INFLUENCE OF SIDEROPHORES AND IRON ON *MYCOBACTERIUM BOVIS* ISOLATION FROM PATHOLOGICAL MATERIAL

Zavgorodniy A. I., Pozmogova S. A., Bilushko V. V., Kalashnyk M. V., Gologurska O. I.

National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: nick.v.kalashnik@gmail.com

Summary. The article presents the results of studying the effect of siderophores and iron on the isolation of *Mycobacterium bovis* from pathological material. It has been established that the simultaneous presence of iron and siderophore from *M. phlei* in the nutrient medium makes it possible to detect the growth of *M. bovis* from pathological material 6–8 days earlier; ensures the growth of more colonies and bacterial mass. The presence of heterologous to mycobacteria siderophore (from *Nocardia* spp.) in the medium reduces the elective (growth) properties of the medium. Siderophores found in the culture filtrate or alcoholic extract of *M. phlei* can be valuable additives to culture media for the accelerated isolation of *M. bovis* from pathological material

Keywords: *Mycobacterium phlei*, *Nocardia asteroides*, nutrient media, elective properties

Introduction. Laboratory diagnostics based on the direct isolation of the pathogen from biological material is one of the priority areas in tuberculosis control. Despite the existence of methods for tuberculosis diagnosing, the culture method retains its significance and is the 'gold' standard for isolating and studying the pathogen. However, *in vitro* cultivation of mycobacteria significantly loses its diagnostic value due to the long periods of generation of the mycobacterium tuberculosis complex. This is due to the peculiarities of the metabolism of mycobacteria and the complex structure of the cell wall, which is relatively impermeable for the exchange of metabolites between the bacterium and its host during chronic intracellular infection (Lyamin et al., 2016; Dobin, Demikhov and Zharikova, 2016).

For successful reproduction in the host organism, pathogens need iron, which takes part in the basic metabolic processes of mycobacteria, including respiration, the Krebs cycle, oxygen transport, gene regulation, protection against oxidative stress, and DNA biosynthesis (Rodriguez, 2006; Arnold et al., 2020; Andrews, Robinson and Rodríguez-Quiñones, 2003).

However, in the tissues of the macroorganism, the concentration of this metal is very low, since iron Fe^{+3} is sequestered by iron-binding proteins such as transferrin, lactoferrin, ferritin, hemopexin, and haptoglobin (Hood and Skaar, 2012; Takatsuka et al., 2011; Dobryszycka, 1997; Tolosano and Altruda, 2002; Jones and Niederweis, 2011; Tullius et al., 2011; Drakesmith and Prentice, 2012).

Mycobacteria have developed both general and specialized systems to transport iron molecules through cell walls (DiGiuseppe Champion and Cox, 2007; Agoro and Mura, 2019).

Iron production mechanisms include: synthesis of siderophores together with siderophore-based transport systems; the acquisition of metal directly from the host's iron-binding proteins (transferrin and lactoferrin); absorption and utilization of heme; dissolution of iron oxides by reduction of iron oxide and transport of soluble forms (Tanner et al., 2017).

Mycobacteria synthesize three types of siderophores to bind and transport iron — mycobactin, carboxymycobactin, and exochelin. The mycobactin molecule is associated with the cell wall of mycobacteria and provides the transfer of iron into the cell, while carboxymycobactin and exochelin are secretory molecules. Saprophytic mycobacteria, living in the soil, synthesize and secrete mainly exochelin, while pathogenic mycobacteria — carboxymycobactin (Fang et al., 2015).

Mutants with impaired mycobactin biosynthesis lose the ability to replicate *in vivo* and *in vitro* (Reddy et al., 2013; Rodriguez and Smith, 2006; Knobloch et al., 2020). In addition to mycobacteria, siderophores are also synthesized by closely related to mycobacteria *Nocardia* spp. (nocabactin) (Hoshino et al., 2011; Dhakal et al., 2019; Männle et al., 2020).

When conditions change from *in vivo* to *in vitro*, mycobacterium tuberculosis temporarily loses the ability to independently synthesize some substances necessary for generation and growth *in vitro*. Therefore, additional time is required for *M. bovis* to adapt to other conditions, different from laboratory-adapted strains. Rich in a variety of bioavailable organic and inorganic nutrients nutrient media are required for the generation and growth of pathogenic mycobacteria.

The research aimed to study the effect of siderophores and iron on the isolation of the tuberculosis pathogen from biological material and to determine the effectiveness of culture media with different growth factors in the isolation and cultivation of *M. bovis*.

Materials and methods. The research used reference cultures of *M. bovis* strain *Vallee* and *M. phlei*, which are stored in the mycobacteria collection of the laboratory for the study of tuberculosis of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine).

A field isolate of *Nocardia asteroides* was isolated from cattle lymph nodes.

The research was carried out using biological and cultural methods.

Infection of laboratory animals. Guinea pigs (n = 3) were infected with a suspension of *M. bovis,* strain *Vallee*, at a dose of 1.0 mg of bacterial mass in 1.0 cm^3 of sterile isotonic NaCl solution. After 30 days, the guinea pigs were euthanized, and the liver and spleen were collected for cultural research.

Pre-plating treatment and plating of pathological material. The biomaterial (liver, spleen) was rubbed with sterile sand, and 0.85% NaCl solution was added to the homogenate in a ratio of 1:3. After precipitation of sand and large particles, the supernatant was collected and centrifuged. The precipitate was decontaminated with 6% sulfuric acid for 20 min and then washed by centrifugation in 0.85% NaCl solution. The sediment of the biological material was resuspended in a small amount of 0.85% NaCl and seeded 0.3 cm^3 on each nutrient medium (n = 5). The cultures were cultivated at a temperature of 37.5 ± 0.5°C for one month.

The rate of appearance of the first colonies, the intensity of growth (the number and size of colonies), and the amount of bacterial mass served as indicators of the effect of various additives on the *M. bovis* growing.

Preparation of culture media. Based on the 'Dry nutrient medium for the cultivation of mycobacteria' we prepared five versions of egg media with various additives. Medium I contained 25% of the culture filtrate of the liquid medium, on which *M. phlei* and ammonium citrate iron were cultivated at a concentration of 0.0075 g per 100.0 cm^3, medium II — 2% ethanol extract of dry bacterial mass *N. asteroides* and citric ammonium iron at a concentration of 0.0075 g per 100.0 cm^3, medium III — 2% ethanol extract of dry bacterial mass of *M. phlei* and citric-ammonium iron at a concentration of 0.0075 g per 100.0 cm^3, medium IV —2% ethanol extract of dry bacterial mass *M. phlei* (without ammoniacal iron), medium V — control (without siderophores and iron). All additives were added just before the coagulation of the medium.

Preparation of M. phlei culture filtrate and ethanol extracts of M. phlei and N. asteroides. The culture of *M. phlei* and *N. asteroides* was cultivated for 4 weeks in bottles with a liquid medium containing asparagine, potassium phosphate, sodium phosphate, sodium citrate, magnesium sulfate, ammonium ferrous sulfate, and glucose. Culture was inactivated by autoclaving at 132°C for 60 min. The bacterial mass of *M. phlei* and *N. asteroides* was separated from the liquid part of the medium by filtration through a double paper filter. The culture filtrate of *M. phlei* was sterilized. The bacterial mass of *M. phlei* and *N. asteroides* was dried, ground into powder, and extracted in ethanol in an oven at 90°C.

Statistical analysis was performed by counting colonies, determining the arithmetic mean and standard error (M ± m).

Results and discussion. The autopsy of guinea pigs 30 days after infection with a suspension of *M. bovis* strain *Vallee* revealed enlarged inguinal lymph nodes and characteristic tuberculous lesions in the form of single grayish-white small nodules in the spleen and liver, which indicated the pathogenicity of the strain. From the results of the culture study, we found that the addition of various siderophores and ammonium-lemon iron to the nutrient medium has a positive effect on the rate and intensity of growth (Figs. 1–4).

The results of a comparative study of the effectiveness of nutrient media in the isolation of mycobacteria from biomaterial are presented in Table 1.

As can be seen from the data in Table 1, when isolating *M. bovis*, the *Vallee* strain from biological material from infected guinea pigs, the nutrient medium I with the addition of *M. phlei* culture filtrate and citric ammonium iron had the best growth qualities, namely, in terms of the rate of appearance of visible colonies and their number.

So, on the surface of the bevels of this medium, the visible growth of the first single colonies less than 0.5 mm in size was noted in 2 tubes on the 7^{th} day of cultivation, the growth in all tubes was detected on the 11^{th} day of incubation.

The research results indicate that the growth rate of the primary colonies of the nutrient medium I is superior to the rest of the mediums.

On medium I, compared with the rest of the studied media, the growth of the causative agent of tuberculosis (in all test tubes) was noted seven days earlier. On media with iron and ethanol extracts of *M. phlei* (III) and *N. asteroides* (II), on 15^{th} day, in 3 tubes of each medium, single colonies (less than 0.5 mm) were detected although on medium III in one test tube on 11^{th} day, the growth of one colony was noted. Regarding medium IV, which included only *M. phlei* extract (without iron) and control medium V, the growth of 1–2 colonies was observed in one tube of each medium on 15^{th} day. We noted the presence of colonies on media II, III, IV, V in all test tubes at the same time — on 18^{th} day.

Differences were also in terms of the intensity of growth of colonies (number, size, amount of bacterial mass). After 30 days of cultivation, the average number of colonies (M ± m) on medium I was 90.0 ± 6.8, which is 2.1 times more than the number of colonies on control medium V (43.2 ± 3.4). The average number of colonies on medium III was 78.2 ± 4.6, then medium II with 65.8 ± 5.2 and medium IV with 52.8 ± 4.4 colonies, which, respectively, 1.8, 1.5 and 1.2 times more than the number of colonies on the control medium.

In addition, as can be seen from Table 1, the amount of bacterial mass on the medium I with the culture filtrate *M. phlei* and iron, compared with the rest of the media, was also greater and amounted to 60.0 ± 3.5 mg, which is 1.6, 1.2, 1.27, 1.33 times more than 'raw' weight of the bacterial mass from medium II (37.5 ± 2.7 mg), medium III (49.3 ± 3.1 mg), medium IV (47.0 ± 3.6 mg) and control medium (45.0 ± 3.4 mg), respectively. It is important to note that on the control medium V, despite the smaller number of colonies (43.2 ± 3.4), their size after 30 days of cultivation was significantly larger than on all other media, which affected the weight of the bacterial mass (45.0 ± 3.4 mg).

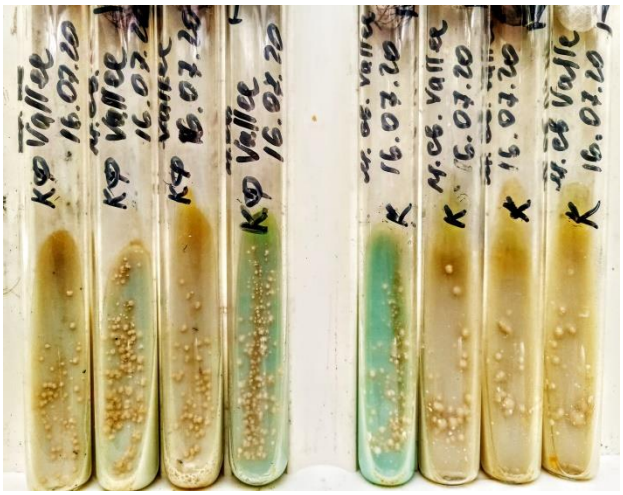

Figure 1. Growth of strain *Vallee* on media I and V (control)

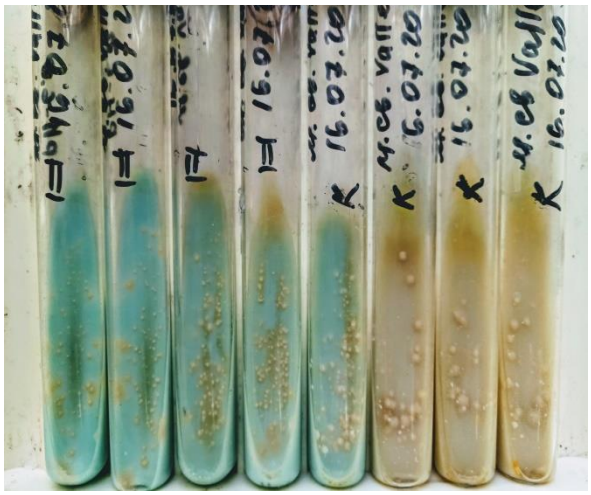

Figure 2. Growth of strain *Vallee* on media II and V (control)

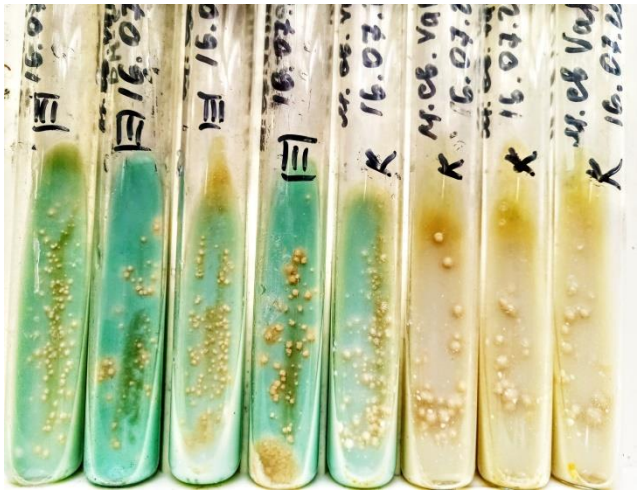

Figure 3. Growth of strain *Vallee* on media III and V (control)

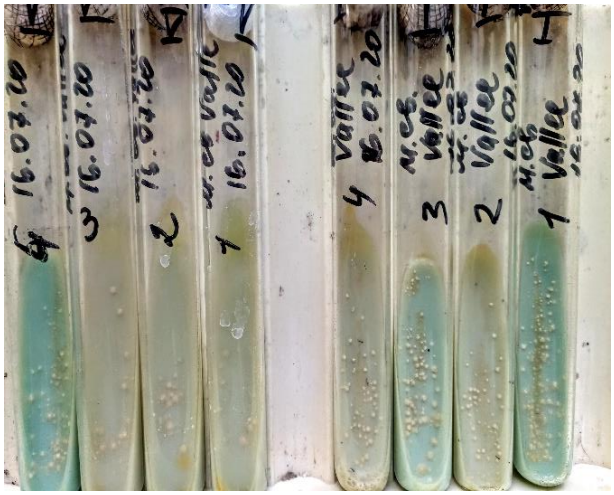

Figure 4. Growth of strain *Vallee* on media IV and V (control)

Table 1 — Results of studying the effect of siderophores and iron in isolating *M. bovis* strain *Vallee* from biological material

Medium	No. of the test tube	Days of cultivation							Number of colonies, M ± m	Bacterial mass weight, mg/test tube, M ± m
		7	11	15	18	20	25	30		
		Number of colonies								
I (CF + Fe)	1	1	3	10	16	36	72	102	90.0 ± 4.6	60.0 ± 3.5
	2	–	1	5	12	22	48	79		
	3	–	2	7	14	29	52	84		
	4	–	1	4	10	19	46	86		
	5	2	4	6	13	22	68	99		
II (ExNoc + Fe)	1	–	–	4	6	16	38	74	65.8 ± 5.2	37.5 ± 2.7
	2	–	–	–	3	8	46	80		
	3	–	–	3	7	13	25	55		
	4	–	–	2	8	12	27	54		
	5	–	–	–	4	9	33	66		
III (ExPhl + Fe)	1	–	–	–	3	11	42	95	78.2 ± 4.6	49.3 ± 3.1
	2	–	–	2	6	14	33	74		
	3	–	1	3	6	12	38	69		
	4	–	–	1	5	13	39	71		
	5	–	–	–	4	10	33	82		

Continuation of Table 1

Medium	No. of the test tube	Days of cultivation							Number of colonies, M ± m	Bacterial mass weight, mg/test tube, M ± m
		7	11	15	18	20	25	30		
		Number of colonies								
IV (ExPhl)	1	–	–	–	4	7	24	48	52.8 ± 4.4	47.0 ± 3.6
	2	–	–	–	3	8	27	40		
	3	–	–	2	10	21	41	62		
	4	–	–	–	6	11	36	59		
	5	–	–	–	5	9	31	55		
V (control)	1	–	–	–	3	8	26	42	43.2 ± 3.4	45.0 ± 3.4
	2	–	–	–	6	16	28	43		
	3	–	–	–	4	12	21	34		
	4	–	–	1	12	35	35	45		
	5	–	–	–	6	12	43	52		

Notes: CF — culture filtrate *M. phlei*; ExNoc — ethanol extract *N. asteroides*; ExPhl — ethanol extract *M. phlei*; Fe — citric ammonium iron.

The ratio of the number of colonies to the weight of the bacterial mass on medium V was less than one, while on medium I, this figure was 1.5, on medium II — 1.7, on medium III —1.6, on medium IV — 1.1.

Thus, from the results of a comparative study of the effectiveness of culture media, it follows that the inclusion of a homologous (mycobacterial) siderophore and iron in the composition makes it possible to improve the quality of the culture medium, and, on the contrary, the addition of heterologous nocabactin does not stimulate the growth of the bacterial mass of *M. bovis*. These data are consistent with studies by other authors.

It was reported that a phytosiderophore extracted from plant roots inhibits the growth of the *Mtb* H37Ra strain *in vitro* (Rajiv et al., 2001). According to Gokarn and Pal (2017), *M. smegmatis* exocholine at a dose of 0.5 mg/ml selectively inhibited the growth of *M. tuberculosis*. An important observation was that the inhibitory activity of siderophores was canceled when iron was added to the medium. This proves the bacteriostatic effect of heterologous siderophores, which is solely due to their ability to deprive pathogens of iron (Gokarn and Pal, 2017; He and Xie, 2011).

This is since the fact that the Fe^{3+} ion forms a complex with a heterologous relative to *Mycobacterium* siderophore (in this case, nocabactin); the absence of specific receptors in mycobacteria will affect the absorption of iron and reduce its availability, which ultimately affects the growth of the pathogen. This statement is confirmed by the data in Table 1, on a medium with an extract of *N. asteroides* and iron (medium II), the weight of the bacterial mass was the smallest.

The source of iron for mycobacteria in the macroorganism is the host metalloproteins (transferrin, ferritin, lactoferrin, hemproteins, primarily hemoglobin, as well as heme-containing enzymes) (Hood and Skaar, 2012).

In vitro, the absence of this most important metal affects the replication rate and growth of mycobacterial cells when plating pathological material. In addition, under aerobic conditions, iron is present in the medium in an oxidized form, Fe (III), forming practically insoluble iron oxide hydrates, iron carbonate, and magnetite.

To transfer iron from the medium to the cell, siderophores, organic compounds that form chelates with Fe^{3+} are necessary. They allow the microbial cell to absorb iron in the complexes with the siderophore and actively grow. Table 1 shows this fact. The simultaneous presence of iron and mycobacterial siderophore in the medium ensured the growth of more colonies and the accumulation of bacterial mass. In addition, vice versa, the absence of iron and siderophore in the medium (control medium V) or one component (medium IV) reduced the growth qualities of the medium at the isolation of the pathogen from pathological material.

The culture filtrate is a variety of *M. phlei* metabolic and autolysis products, including siderophores (mycobactin and exochelin), which mediate the iron absorption by mycobacteria.

It should be noted that pathogenic mycobacteria do not form exochelin peptide siderophore synthesized by non-pathogenic mycobacteria (in this case *M. phlei*), but utilize it. Apparently, in the culture filtrate, the substances necessary for the growth of *M. bovis* are found in a greater amount and in more bioavailable form than in the alcohol extract of *M. phlei*. The results of the study show that the presence of exochelin and other products of metabolism and autolysis of *M. phlei* contained in the culture filtrate, in comparison with other growth factors, contribute to the accelerated indication of *M. bovis* from biological material.

The next stage of our research was to study the growth activity of subcultures of *M. bovis*, strain *Vallee* of the first generations in the absence of siderophores in the medium. For this, from each medium, a suspension of the first generation of grown cultures was inoculated on a nutrient medium without siderophore and iron (Table 2).

Table 2 — The intensity of growth of *M. bovis* subcultures of the first generation on a nutrient medium without iron and siderophores

Subculture	No. of test tube	Days of cultivation						Bacterial mass weight, mg/test tube, M ± m
		5	6	8	10	15	20	
		Number of colonies						
From medium I (CF + Fe)	1	–	+	++	++	+++	++++	91.1 ± 3.1
	2	–	–	+	++	+++	++++	
	3	–	+	++	++	+++	++++	
	4	–	+	+	+	++	++++	
	5	–	+	+	+	++	++++	
From medium II (ExNoc + Fe)	1	–	–	+	+	++	+++	77.4 ± 4.2
	2	–	+	+	++	+++	++++	
	3	–	–	+	++	++	+++	
	4	–	–	+	+	+	++	
	5	–	–	+	+	+	++	
From medium III (ExPhl +Fe)	1	–	+	++	++	+++	++++	89.8 ± 3.1
	2	–	–	++	++	+++	++++	
	3	–	–	+	+	++	++++	
	4	–	+	+	++	++	++++	
	5	–	–	+	+	++	++++	
From medium IV (ExPhl)	1	–	–	+	+	++	++++	89.3 ± 2.7
	2	–	+	+	+	++	++++	
	3	–	+	+	++	+++	++++	
	4	–	+	++	+	+++	++++	
	5	–	–	+	+	++	++++	
From medium V (control)	1	–	+	++	++	+++	++++	88.8 ± 3.8
	2	–	+	+	++	+++	++++	
	3	–	+	++	++	+++	++++	
	4	–	–	+	+	++	++++	
	5	–	–	+	+	++	++++	

Notes: (-) — no colonies; (+) — up to 10 colonies; (++) — from 10 to 30 colonies; (+++) — from 30 to 50 colonies; (++++) — more than 50 colonies; CF — cultural filtrate *M. phlei*; ExNoc — ethanol extract *N. asteroides*; ExPhl — ethanol extract *M. phlei*; Fe — citric ammonium iron.

As a result of the cultural study, it was found that, regardless of the composition of the medium on which the culture was originally isolated, when subcultured to media without iron and siderophore, the first colonies of subcultures were observed at the same time on the 6^{th}–8^{th} days with a similar growth rate.

In addition, the culture grown on the control medium V, which initially did not include siderophores and iron, showed an identical growth of colonies during subculturing and at the same time as on the other media.

Mycobacteria of tuberculosis complex regulate iron metabolism at the level of gene transcription, inducing the expression of genes for iron uptake in case of iron deficiency and activating its storage when it is readily available through the function of an iron-dependent regulator to turn off iron absorption and turn on its conservation (Rodriguez et al., 2002; Gupta et al., 2009).

The absence of growth factors and iron in the nutrient medium did not have a negative effect on the rate and intensity of subculture growth. This was due to the fact that the iron accumulated by mycobacteria through the siderophores served as a reserve for overcoming its deficiency, and the subculture that grew on the control medium (without iron and siderophore) managed to adapt.

Therefore, the absence of iron and siderophores from *M. phlei* in the medium also did not have a particular effect on the accumulation of bacterial mass, its amount ranged from 89.3 ± 2.7 mg to 91.1 ± 3.1; a smaller amount of bacterial mass was obtained from the subculture, originally grown on medium II (with ethanol extract of *N. asteroides* + Fe).

Conclusions. Since the work was of a research nature, we studied the effect of siderophores on the rate and intensity of growth on one pathogen. However, it has provided a 'proof of concept' that siderophores found in culture filtrate or alcoholic extract of saprophytic mycobacteria can be valuable supplements in culture media for isolating tuberculosis pathogens from pathological material.

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Part 3. Biosafety

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DEVELOPMENT OF 'BONDARMINE' DISINFECTANT FORMULATION AND STUDY OF ITS TUBERCULOSIS EFFECT

Bondarchuk A. O. [1], Pavlichenko O. V. [1], Zavgorodniy A. I. [2], Paliy A. P. [2]

[1] Kharkiv State Zooveterinary Academy, Kharkiv, Ukraine, e-mail: ancheystar777@ukr.net
[2] National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: paliy.dok@gmail.com

Summary. The article presents the results of studying the bactericidal properties of the disinfectant 'Bondarmin', consisting of potassium peroxymonosulfate, sulfonol (surfactant), adipic acid (hexanedioic acid), and sodium chloride. The active component of the preparation is potassium peroxymonosulfate ($KHSO_5$), which being HSO_5 ion in solution, has an oxidizing effect on the cysteine moieties of microorganism proteins. The highest oxidation-reduction potential is achieved in an acidic medium (pH 2.0–2.3), which is provided by a buffer additive — adipic acid, as well as additionally the presence of sodium chloride in the solution, which creates a high ionic force of the solution, and therefore contributes to the high osmotic pressure of the solution — important factors of biocidal action. $KHSO_5$ slowly oxidizes chlorides to chlorine, which has an additional bactericidal effect (high availability of disinfectant to internal surfaces). Studies on the bactericidal properties of the 'Bondarmin' disinfectant were carried out in accordance with the methodological recommendations 'Determination of bactericidal properties of disinfectants, disinfection and control of its quality in tuberculosis of farm animals'. Experimental studies have established bactericidal effect of disinfectant 'Bondarmin' in concentration 1.0% per exposure of 3 hr in relation to atypical mycobacteria *M. fortuitum* and tuberculosis pathogens *M. bovis* and *M. avium*

Keywords: mycobacteria, test objects, exposure, concentration, bactericidal action

Introduction. Animal husbandry today is a branch of the industrial complex of Ukraine, which is characterized by high efficiency and technological efficiency and is able to provide the population with high-quality food products in a minimum period of time (Kovalenko et al., 2020).

Along with this, diseases of infectious etiology cause significant losses to farms, which negatively affects the epizootic situation and the economy of enterprises (Mustapha et al., 2018).

Along with this concentration of livestock in a limited area, it conditions favorable conditions for the emergence and spread of diseases of contagious etiology. The course of diseases in livestock leads to a decrease and loss of productivity, decrease in animal body weight gain, death, additional financial costs for therapeutic and preventive measures, deterioration in the safety and quality of livestock products (Bondarchuk, Paliy and Blazheyevskiy, 2019, Saccucci et al., 2018). Measures to combat infectious diseases can only be effective if the epizootic chain is broken, so the development and introduction of new disinfectants is an urgent task of veterinary science (Nechyporenko et al., 2019).

Disinfectants should have high biocidal capacity, prolonged action, reduced aggressiveness to equipment and treated surfaces, safety for personnel and the environment, ease of use, moderate price (Rutala and Weber, 2016). Over time, microorganisms develop resistance to disinfectants. Systematic rotation of desiccants based on various active substances can effectively prevent this phenomenon (Kanishchev and Eremeeva, 2016).

It should be noted that today a large number of biocides are used in veterinary practice, which have differences in the form of release and chemical components. The selection of disinfectants is carried out, taking into account the method of keeping animals and the type of livestock room, while the type of floor of the room is necessarily taken into account (Shkromada et al., 2019). Thus, based on the above-mentioned, the development and use of the latest disinfectants with an expanded spectrum of their antimicrobial activity by combining active components, their ability to prevent the occurrence of resistance in bacteria and viruses is a pressing issue (Paliy et al., 2020b, 2021). It should be emphasized that working solutions of new combinations of desiccants should have the property of use in the presence of animals. First of all, this is important because disinfecting into this method will reduce the total bacterial contamination of livestock premises and improve microclimate parameters and veterinary and sanitary conditions for animals and poultry in general (Paliy et al., 2020a).

The aim of the research was to develop a new disinfectant 'Bondarmin' for wet disinfection of livestock premises and veterinary facilities.

Material and methods. The study was carried out in accordance with the methodological recommendations 'Determination of bactericidal properties of disinfectants, disinfection and control of its quality in tuberculosis of

farm animals' (Zavhorodnii et al., 2007). At the beginning of the studies, the bactericidal effect of an aqueous solution of chemical substances on atypical mycobacteria of the species *M. fortuitum* was established.

The next step was establish the bactericidal action of the disinfectant on various test objects contaminated with 2 billion veils separately with *M. bovis* and *M. avium* mycobacteria. As test objects, batiste, unpainted wood, tile, metal, plastic, and glass were used. Sterile pus (as a biological defense against chemical factors) was also added to the dependence of mycobacteria at a rate of 2:1. The infective material was evenly distributed on the surface of each test and control object 'Bondarmin' disinfectant was used at a concentration of 1.0, 1.5, and 2.0% for expositions 1, 3, 5, and 12 hrs.

A final study of the bactericidal properties of the disinfectant against *M. fortuitum, M. bovis*, and *M. avium* was carried out by bioprobe. In order to carry out this study, we selected 30 guinea pigs with an average weight of 330 ± 20 g and 12 rabbits with an average weight of 2.5 ± 0.2 kg. All animals were tested using tuberculin (PPD) for mammals at a dose of 0.1 ml.

After a negative response was determined in animals to the introduction of the allergen, 7 groups (3 research and 4 control) were formed from them (each group had 6 animals):

I research group (guinea pigs) — animals were injected subcutaneously with flushes (1.0 cm^3) from test objects (composite sample) contaminated with the pathogen of tuberculosis *M. bovis* and treated with disinfectant 'Bondarmin' (1.0%, 3 hrs);

II research group (rabbits) — animals were injected subcutaneously with flushes (1.0 cm^3) from test objects (composite sample) contaminated with the pathogen of tuberculosis *M. avium* and treated with disinfectant 'Bondarmin' (1.0%, 3 hrs);

III research group (guinea pigs) — animals were injected subcutaneously (1.0 cm^3) with fast-growing atypical mycobacteria *M. fortuitum* and treated with disinfectant 'Bondarmin' (1.0%, 3 hrs);

IV control group (guinea pigs) — animals were injected subcutaneously with flushes (1.0 cm^3) from test objects (composite sample) contaminated with the pathogen of tuberculosis *M. bovis* and treated with sterile saline;

V control group (rabbits) — animals were injected subcutaneously with flushes (1.0 cm^3) from test objects (composite sample) contaminated with the pathogen of tuberculosis *M. avium* and treated with sterile saline;

VI control group (guinea pigs) — animals were injected subcutaneously (1.0 cm^3) with fast-growing atypical mycobacteria *M. fortuitum* and treated with sterile saline;

VII control group (guinea pigs) — intact animals.

Laboratory animals were observed for three months, once a month they were examined by an allergic method for tuberculosis, by using mycobacterial allergens. The animals of groups I, IV, VII were intracorally injected with tuberculin (PPD) for mammals at a dose of 0.1 cm^3; II, V — tuberculin (PPD) for poultry at a dose of 0.1 cm^3; III, VI, VII — allergen from atypical mycobacteria (AAM) at a dose of 0.1 cm^3.

Results and discussion. To create a new disinfectant, we studied and analyzed a number of chemical ingredients. At the same time, the most promising substances were identified in our opinion: allicin, cetylpyridinium chloride, potassium peroxymonosulfate, on the basis of which it is possible to create an innovative disinfectant.

In order to determine their potential for use as an active substance in a new disinfectant, we conducted studies to determine their bactericidal effect on the test culture of atypical mycobacteria *M. fortuitum* (strain No. 122). Experimental studies were carried out using the culture method of studies (Table 1).

Table 1 — Bactericidal properties of chemicals to *M. fortuitum* (strain No. 122)

Application mode, %/hrs	Results	
	experience	control
Potassium peroxymonosulfate		
0.5/3	+	+
0.5/5	+	+
0.5/24	+	+
1.0/3	–	+
1.0/5	–	+
1.0/24	–	+
1.5/3	–	+
1.5/5	–	+
1.5/24	–	+
2.0/3	–	+
2.0/5	–	+
2.0/24	–	+
Cetylpyridinium chloride		
0.5/3	+	+
0.5/5	+	+
0.5/24	+	+
1.0/3	+	+
1.0/5	+	+
1.0/24	+	+
1.5/3	+	+
1.5/5	+	+
1.5/24	+	+
2.0/3	+	+
2.0/5	+	+
2.0/24	+	+
Allicin		
0.5/3	+	+
0.5/5	+	+
0.5/24	+	+
2.0/3	+	+
2.0/5	+	+
2.0/24	+	+

Notes: '–' — there is no growth of mycobacteria, '+' — the existing growth of mycobacteria.

From the results of Table 1, it can be seen that potassium peroxymonosulfate has bactericidal properties with respect to *M. fortuitum* at a concentration of 1.0% per exposure of 3 to 24 hrs. In addition, the test powders, such as cetylpyridinium chloride and allicin did not show bactericidal effect to the mycobacteria test culture when tested at a concentration of up to 2.0% per action for 24 hrs, and therefore their further use as active substances is not appropriate.

According to the results of the studies, potassium peroxymonosulfate turned out to be an effective bactericide in the test culture of mycobacteria. This substance has been identified by us as the active ingredient for the further design of the new disinfectant.

In order to improve the physicochemical properties of this compound and reduce its content in the preparation, the adjuvants shown in Table 2 were added to the formulation of the new agent.

Table 2 — Disinfectant research formulations (working solutions)

No.	Composition	Content
1	Potassium peroxymonosulfate	1.0%
	Sulfonol	0.1%
	Adipic acid	0.5%
	Sodium chloride	0.4%
	Water	to 100%
2	Potassium peroxymonosulfate	2.0%
	Sulfonol	0.2%
	Adipic acid	1.0%
	Sodium chloride	0.8%
	Water	to 100%

The presence of bactericidal action compositions of chemical compounds (Table 2) was determined in tests with *M. fortuitum* test culture (No. 122). The results of the experiment are shown in Table 3.

Table 3 — Bactericidal action of chemical compounds to *M. fortuitum*

Chemical compounds	Exposition	Results	
		experience	control
No. 1	10 min	+	+
	30 min	+	+
	1 hr	+	+
	2 hr	+	+
	3 hr	–	+
	4 hr	–	+
	5 hr	–	+
No. 2	10 min	+	+
	30 min	+	+
	1 hr	+	+
	2 hr	+	+
	3 hr	–	+
	4 hr	–	+
	5 hr	–	+

Notes: '–' — there is no growth of mycobacteria, '+' — the existing growth of mycobacteria.

From the results shown in Table 3 it can be seen that these chemical compounds, when combined in these ratios, exhibit a bactericidal effect on *M. fortuitum* mycobacteria starting from exposure of 3 hrs or more.

So, after determining the compatibility of these chemical compounds, the most optimal ratio of their contents in the new disinfectant was calculated.

Thus, the new disinfectant consists of: potassium peroxymonosulfate — 50.0%, sulfonol — 5.0%, adipic acid — 25.0%, sodium chloride — 20.0%. This composition was called 'Bondarmin' by us and taken for further experimental studies.

The study of the bactericidal properties the disinfectant 'Bondarmin' was carried out using a suspension method using a test culture of *M. fortuitum* mycobacteria (strain No. 122). Working solutions of the disinfectant were prepared just before the start of the studies in sterile vials. Disinfectant 'Bondarmin' was examined at a concentration of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% for the drug when exposed to 1, 3, 5, and 24 hrs. The results of the studies are shown in Table 4.

Table 4 — Bactericidal properties of 'Bondarmin' disinfectant in solution to *M. fortuitum* (n = 5)

Concent-ration, %	Exposition, hrs					Control	
	1	3	5	12	24	negative	positive
0.5	+	+	+	+	+	–	+
1.0	+	–	–	–	–	–	+
1.5	+	–	–	–	–	–	+
2.0	–	–	–	–	–	–	+
2.5	–	–	–	–	–	–	+
3.0	–	–	–	–	–	–	+

Notes: '–' — there is no growth of mycobacteria, '+' — the existing growth of mycobacteria.

From the results given in Table 4, it can be seen that the disinfectant 'Bondarmin' in the concentration 0.5% by exposure from 1 to 24 hrs does not bactericidally affect the test culture of *M. fortuitum*, while an increase in the concentration of the agent up to 1.0–1.5% per action from 3 hrs predetermines the death of mycobacteria. High bactericidal properties with respect to atypical fast-growing mycobacteria, the test agent exhibits for actions in concentration 2.0–3.0% and exposure from 1 to 24 hrs.

The positive results obtained give us reason to continue studying the bactericidal properties of the developed disinfectant directly with the pathogens of tuberculosis of animals and birds.

The next stage of our research was the study of the bactericidal properties of disinfectant on test objects with *M. bovis* and *M. avium* cultures. It is necessary to conduct studies directly with tuberculosis pathogens, bringing the experimental conditions closer to production conditions. To this end, we have determined the physical nature of the materials used in animal husbandry. The results of the studies are presented in Table 5.

Table 5 — Bactericidal properties of 'Bondarmin' disinfectant to test objects with cultures *M. bovis* (n = 3) and *M. avium* (n = 3)

Test culture	Application mode, %/hrs	Test objects						Control	
		B	W	T	M	P	G	negative	positive
Mycobacterium bovis (Vallee strain)	1.0/1	+	+	+	+	+	+	–	+
	1.0/3	–	–	–	–	–	–	–	+
	1.0/5	–	–	–	–	–	–	–	+
	1.0/12	–	–	–	–	–	–	–	+
	1.5/1	+	+	+	+	+	+	–	+
	1.5/3	–	–	–	–	–	–	–	+
	1.5/5	–	–	–	–	–	–	–	+
	1.5/12	–	–	–	–	–	–	–	+
	2.0/1	–	–	–	–	–	–	–	+
	2.0/3	–	–	–	–	–	–	–	+
	2.0/5	–	–	–	–	–	–	–	+
	2.0/12	–	–	–	–	–	–	–	+
Mycobacterium avium (IECVM UAAS strain)	1.0/1	+	+	+	+	+	+	–	+
	1.0/3	–	–	–	–	–	–	–	+
	1.0/5	–	–	–	–	–	–	–	+
	1.0/12	–	–	–	–	–	–	–	+
	1.5/1	+	+	+	+	+	+	–	+
	1.5/3	–	–	–	–	–	–	–	+
	1.5/5	–	–	–	–	–	–	–	+
	1.5/12	–	–	–	–	–	–	–	+
	2.0/1	–	–	–	–	–	–	–	+
	2.0/3	–	–	–	–	–	–	–	+
	2.0/5	–	–	–	–	–	–	–	+
	2.0/12	–	–	–	–	–	–	–	+

Notes: '–' — there is no growth of mycobacteria, '+' — the existing growth of mycobacteria; B — batiste, W — unpainted wood, T — tile, M — metal, P — plastic, G — glass.

Statistical processing of the obtained 'Bondarmin' disinfectant regimens was performed using non-parametric statistical criteria (Z mark). To this end, the results of studies of a single experiment with *M. bovis* culture, performed in triplicate, in which the mode of use of the disinfectant was 1.0% per exposure of 3 hrs, were taken into account. The results of the studies are shown in Table 6.

According to the results of the data given in Table 6, it can be stated that the disinfectant 'Bondarmin' inactivates the test culture of the tuberculosis pathogen *M. bovis* when used at a concentration of 1.0% per exposure for 3 hrs, regardless of the physical nature of the test object, with a probability of 99%, which indicates the prospect of using this drug as a tuberculosis disinfectant.

Table 6 — Statistical processing of data on studies of bactericidal action of disinfectant 'Bondarmin' (1.0%, 3 hrs)

Test object, quantity		Growth of mycobacteria colonies on nutrient medium				Criterion Z
		to action (add)	after action (add)	control		
				negative	positive	
1	B	+	–	–	+	positive
2		+	–	–	+	positive
3		+	–	–	+	positive
1	W	+	–	–	+	positive
2		+	–	–	+	positive
3		+	–	–	+	positive
1	T	+	–	–	+	positive
2		+	–	–	+	positive
3		+	–	–	+	positive
1	M	+	–	–	+	positive
2		+	–	–	+	positive
3		+	–	–	+	positive
1	P	+	–	–	+	positive
2		+	–	–	+	positive
3		+	–	–	+	positive
1	G	+	–	–	+	positive
2		+	–	–	+	positive
3		+	–	–	+	positive

Notes: '–' — there is no growth of mycobacteria, '+' — the existing growth of mycobacteria; B — batiste, W — unpainted wood, T — tile, M — metal, P — plastic, G — glass; criterion Z — a positive effect of the action.

The biological test in determining the tuberculosis regime of the disinfectant is a necessary final step in determining the final mode of disinfectant use in laboratory conditions. The results of the studies are shown in Table 7.

Table 7 — Biological study of tuberculosis action of disinfectant 'Bondarmin' (1.0%, 3 hrs) on guinea pigs and rabbits

Group	Day of research	Laboratory animal number					
		1	2	3	4	5	6
I	30	–	–	–	–	–	–
	60	–	–	–	–	–	–
	90	–	–	–	–	–	–
II	30	–	–	–	–	–	–
	60	–	–	–	–	–	–
	90	–	–	–	–	–	–
III	30	–	–	–	–	–	–
	60	–	–	–	–	–	–
	90	–	–	–	–	–	–
IV	30	+	+	dead	+	+	dead
	60	dead	+	~	dead	dead	~
	90	~	dead	~	~	~	~
V	30	dead	dead	dead	dead	dead	dead
	60	~	~	~	~	~	~
	90	~	~	~	~	~	~

Continuation of Table 7

Group	Day of research	Laboratory animal number					
		1	2	3	4	5	6
VI	30	+	+	+	+	+	+
	60	+	+	+	–	+	+
	90	+	–	–	–	–	–
VII	30	–	–	–	–	–	–
	60	–	–	–	–	–	–
	90	–	–	–	–	–	–

Notes: '–' — there is no reaction to the administration of allergens; '+' — reaction to the administration of allergens is present; '~' — the absence of an animal in the experience.

By analyzing the results of the bioprobe in laboratory animals, determined that the experimental animals, which were administered flushes from test objects after their treatment with the disinfectant 'Bondarmin' in no case reacted to the intracutaneous administration of the corresponding allergens, which indicates the absence of infection of these animals with mycobacteria. Therefore, it is confirmed that these microorganisms are completely inactivated by the disinfectant under investigation.

In addition, in laboratory animals of control groups (IV–VI), the manifestation of allergic reactions of varying intensity to the administration of mycobacterial allergens was noted. Rabbits died from a septic form of tuberculosis.

In control laboratory animals, lesions characteristic of tuberculosis were found at the autopsy. In research laboratory animals, no characteristic tuberculosis lesions were found during autopsy.

Conclusions. A new disinfectant 'Bondarmin' was developed, which included potassium peroxymonosulfate — 50%, sulfanol — 5.0%, adipic acid — 25%, sodium chloride — 20%. Disinfectant 'Bondarmin' exhibits bactericidal properties at a concentration of 1.0% per exposure of 3 hours relative to *M. fortuitum*, *M. bovis*, *M. avium.*

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MONITORING OF THE QUALITY COMPLIANCE OF BOILED SAUSAGES WITH THE REQUIREMENTS OF THE NATIONAL STANDARD AND LEGISLATION

Khimych M. S., Rodionova K. O.

Odessa State Agrarian University, Odessa, Ukraine,
e-mail: khimichms@gmail.com, katerina.rodionova@ukr.net

Summary. It is important to remember that the most important factor in forming the adult health is the proper diet of children. Despite the growing interest in a healthy lifestyle and diet all over the world, society tends to continue buying ready-made food, the use of which is associated with saving time on cooking. In Ukraine, the traditional meat products are sausage products, in particular, sausages. Studies of the diet of modern schoolchildren show that 35% of respondents constantly consume sausages. It is important to take into account that the main circumstance for the benefit of a food product is its safety for human consumption. The purpose of this work has been to analyze the compliance of certain indicators of safety and quality of sausages of the premium grade 'Dytiachi', which are manufactured by various domestic producers, to the requirements of DSTU 4436: 2005 'Boiled sausages, frankfurters, sardellas, meat loaves. General specifications'. The results of the analysis of the labeling of sausages 'Dytiachi' are presented in the article. They are testifying that in accordance with demanding to paragraph 1 of article 6 of the Law of Ukraine 'About information for consumers about food products' the labels contain basic production and consumer information. At the same time, there is no information provided by article 23 of this law on the labels of the samples: namely the mass of saturated fat and salt. It was also found that as a labeling part of the tested samples (No. 1, 2, and 4) the manufacturers use the statement 'Recommended for diet of preschool aged children (from 3 years) and children of school age', which is compliance/consistent with the text of DSTU 4436:2005 'Boiled sausages, frankfurters, sardellas, meat loaves. General specifications', but, contradict to articles 1 and 4 of part 4 of 'Requirements for claims on the nutritional value of foods and claims on the health benefits of foods'. According to the results of the study of the chemical composition, it was found that in the sample No. 2 the mass fraction of table salt was 2.11 ± 0.14%, which exceeds the content regulated by the state standard by 0.11%

Keywords: safety, chemical composition, children diet, bacterial contamination, content of radionuclides

Introduction. The food market globalization has led to the need to solve the problem of food quality and safety and reduce the risks of their negative impact on population health (Kotelevych and Larina, 2020).

Today, providing the population with safe and quality food is of vital social and epidemiological importance, as it affects the quality of life and health of citizens (Paliy et al., 2020).

It is important to remember that the most vital factor in forming the health of an adult is the proper children diet. According to the WHO, 60% of a child's health depends on the quality and safety of food. That is, first of all, the diet of children that affects all processes of development of their body. Physical and mental potential, which is accumulated in childhood, ensure the health of an adult, and formed social and eating habits — assist to the further formation of behavioral patterns for a healthy lifestyle (Hashchuk, Moskaliuk and Fedorko, 2014; Nyankovskyy and Sadova, 2018; Voitsitska, 2019; Merkulova et al., 2020).

Adequate nutrition is necessary for the growth and development of the body, so meat products are important in the human diet. Namely meat contains essential amino acids, B vitamins and vital minerals such as calcium, phosphorus, potassium and magnesium, which are especially important for the formation and maintenance of health in childhood (Bohatko et al., 2017; Buts, 2017; Rodionova and Paliy, 2018; Andryuschenko et al., 2020; Hashchuk, Moskaliuk and Simonova, 2020). However, despite the growing worldwide interest in a healthy lifestyle and diet, taking into account the pace of modern life, society continues to tend to buy ready meals and semi-finished products, the use of which is associated with saving time on cooking (Paliy et al., 2020). In particular, sausages are a traditional product of the food industry in Ukraine. According to the analysis of the modern Ukrainian market, sausages take the fourth place in the scale of products that are in constant demand among the population (Romaniuk, 2019; Paliy et al., 2020; Khimich et al., 2020).

Studies of the schoolchildren diet nowadays show that 45% of them regularly eat industrially produced food and 35% of the respondents mentioned sausages (Nyankovskyy and Sadova, 2018).

It is important to keep in mind that the main condition for the benefit of a food product is its safety for human consumption (Rodionova and Paliy, 2018; Khimich et al., 2020). A consumer is able to appreciate its marketable qualities–appearance and freshness buying a certain meat product. However, the buyer cannot judge the most important characteristic of sausages — environmental and food safety by its appearance. The safety is characterized by the presence in the product of toxic substances, nitrosamines, pesticides, aflatoxin B1, hormonal drugs, radionuclides, etc. (Paliy et al., 2020). In addition, the rapid development of the food industry, the use of modern raw materials and the creation of new methods in food production, along with the economic crisis, have led to the rapid development of food additives and substitutes of natural ingredients in food,

which can pose a danger to a child's health (Yevstafieva et al., 2017; Paliy et al., 2020; Skrypka et al., 2020; Tytarenko, 2020).

In this regard, the control of safety and quality of meat and meat products, especially recommended for children's diet is still relevant (Andryuschenko et al., 2020; Merkulova et al., 2020; Paliy et al., 2020).

The aim of the work has been to analyze the compliance of certain indicators of safety and quality of sausages of the premium grade 'Dytiachi' which are manufactured by various domestic producers, to the requirements of DSTU 4436:2005 'Boiled sausages, frankfurters, sardellas, meat loaves. General specifications' (DSSU, 2006).

Materials and methods. Experimental studies have been conducted on the basis of a multidisciplinary laboratory of veterinary medicine of the Odesa State Agrarian University, in accordance with current regulations: analysis of labeling in accordance with the Law of Ukraine 'About information for consumers about food products' (VRU, 2019); sampling and sensory evaluation of sausages in accordance with DSTU 4823.2:2007 (DSSU, 2008a); determination of chemical components (mass fraction of protein, fat, moisture, sodium chloride) has been done with using the express analyzer FoodScan Lab (Denmark); microbiological parameters have been determined in accordance with DSTU ISO 4833:2006 (ISO 4833:2003, IDT) (DSSU, 2008b) (content of Mesophilic-aerobic and facultative anaerobic microorganisms (MAFAnM)), DSTU ISO 4832:2015 (ISO 4832:2006, IDT) (SE 'UkrNDNC', 2018) (presence of coliform bacteria), DSTU ISO 15213:2014 (ISO 15213:2003, IDT) (SE 'UkrNDNC', 2016) (presence of sulphite-reducing clostridia in 1 g of product), DSTU ISO 6888-2:2003 (ISO 6888-2:1999, IDT) (DSSU, 2005a) (presence of staphylococci in 1 g of product), DSTU EN 12824:2004 (EN 12824:1997, IDT) (DSSU, 2005c) (presence of *Salmonella spp.* in 25 g of product), DSTU ISO 11290-2-2003 (ISO 11290-2:1998, IDT) (DSSU, 2005b) (presence of *Listeria monocytogenes* in 25 g of product); content of ^{137}Cs and ^{90}Sr radionuclides — by β-γ-spectrometry.

The objects of the study have been sausages of the premium grade 'Dytiachi' domestic producers: 'Sample 1' (Dnepropetrovsk Region), 'Sample 2' (Kirovograd Region), 'Sample 3' (Odesa Region), 'Sample 4' (Poltava Region).

Results and discussion. The results of the analysis of the quality and the presence of information on the labels of the experimental sausages are given in Table 1.

From the data of the Table 1 it is seen that the labeling of all test samples contain mandatory information, which is regulated in paragraph 1 of article 6 of the Law of Ukraine 'About information for consumers about food products' (VRU, 2019).

However, article 23 of this law further foresees that mandatory information on the nutritious value of a food product must include 'the content of fats, saturated fats, carbohydrates, sugars, proteins and salt'. However, none of the samples contains information on the mass content of saturated fat and salt, and the carbohydrate content has been indicated only on the label of sample 2.

After analyzing the list of the ingredients, it been determined that all tested samples contain such an ingredient as food additive E 250 (sodium nitrite), the presence of which is approved for usage in the meat processing industry to stabilize the color. At the same time, at this juncture it is well known that nitrites have a negative effect on health: they influence blood hemoglobin, which leads to lower blood pressure, weak heart and lung (especially dangerous for infants, children and people suffering from cardiovascular disease); damage the thyroid activity, and the gland, heart, central nervous system as well; cause poisoning, especially in infants; Nitrates and Nitrites are precursors of nitrate substitutes, which cause liver disease and have carcinogenic properties. In addition, the negative effects of sodium nitrite increase during heating, which is especially relevant king into account the common consumers' habit to cook or fry sausages (Tytarenko, 2020). In this regard, there is still a relevant question about the feasibility and safety of the use of sausages, especially recommended for children, such a food additive. In addition to the mandatory information, the packaging of all the test samples also contain some additional information: statements about the quality and safety of the product. Thus, the labeling of samples No. 1, 3, and 4 indicates that the product is produced 'GMO-free'; sample 2 — 'from selected types of meat Beef 50%/Pork 40%' and 'Soya been-free/Gluten-free', and the labels of samples No. 1, 2, and 4 contain the mark 'Recommended for preschool children (from 3 years) and school age'.

It is the label 'Recommended for nutrition of preschool children (from 3 years) and school age' that raises controversial questions. After all, the use of such a statement in food labeling can create the impression of safety and even the benefits of this product for consumption among the consumers.

The annexes to DSTU 4436:2005 'Boiled sausages, frankfurters, sardellas, meat loaves. General specifications' (DSSU, 2006) (Table A.2) is mentioned that 'Dytiachi sausages' are 'sausages recommended by the Ministry of Health for baby food'.

At the same time, approved by the order of the Ministry of Health of Ukraine 'Requirements for claims on the nutritional value of foods and claims on the health benefits of foods' (hereinafter — the Requirements) (MHU, 2020) do not contain among the lists of permitted claims (Annexes 2–3), and article 1 of part 4 of the Requirements states 'It is prohibited to use health claims that are not contained in Annex 2 or Annex 3'. In addition, article 4 of part 4 of the Requirements states that 'Recommendations or approvals by medical associations, nutritionists' associations and other organizations aimed to improve human health may be applied only if there is scientifically substantiated evidence for such recommendations and approvals'.

Table 1 — Information for consumers about sausages of the premium grade 'Dytiachi' is indicated on the packaging of various manufacturers

Experimental indicator	Samples			
	1	2	3	4
Product name	Sausages 'Dytiachi'			
The document according to which the product is manufactured	DSTU 4436:2005 'Boiled sausages, frankfurters, sardellas, meat loaves. General specifications' (DSSU, 2006)			
List of ingredients	raw meat 90% (pork skimmed semi-fat, beef skimmed the first grade, skimmed beef of the highest grade), refined sunflower oil, chicken eggs, skimmed milk powder, drinking water, table salt, white sugar, ground nutmeg, pepper powder, ascorbic acid (antioxidant), color fixative (sodium nitrite)	meat — 90% (semi-fat pork, first grade beef from young animals, premium beef), drinking water, sunflower oil, egg powder, skimmed milk powder, table salt, sugar, antioxidant sodium ascorbate, ground nutmeg, ground pepper, color stabilizer sodium nitrite	skimmed meat: first grade beef 30%, premium beef 20%, semi-fat pork 40%, refined sunflower oil, chicken eggs, skimmed cow's milk powder, table salt, granulated sugar, ground nutmeg, ground pepper, color stabilizer (sodium nitrite E 250)	raw meat 90% (pork skimmed semi-fat, skimmed beef of the first grade from young animals, skimmed beef of premium grade), drinking water, refined sunflower oil, egg melange, skimmed cow's milk powder, table salt, sugar — on antioxidant isoascorbate, ground nutmeg, ground pepper, color stabilizer — sodium nitrite
The nutritional value of the product, 100 g	protein not less 12.0 g, fats not more 17.0 g	protein not less 12 g, fats not more 17 g, carbohydrates — 0.9 g	proteins 13.86%, fats 17%	protein not less than 12 g, fats not more than 17 g
Energy value of the product, 100 g	201 Kcal/840 kJ	201 Kcal (841 kJ)	201 Kcal	841 kJ (201 Kcal)
Storage conditions and shelf life	At relative humidity not higher than 75–78% at temperatures from 0 to 6°C, packed in the conditions of the modified gaseous environment: no more than 12 days, provided the integrity of the package	Packed under vacuum. Storage conditions at temperatures from 0 to 6°C and relative humidity of 70–80% — 15 days. After opening the package, use during 48 hours within the expiration date	In a protective environment the gas mixture. Store at temperatures from 0 to 6°C, humidity of 75–78%	If stored in an airtight container for 10 days at a temperature of 0 to 6°C and relative humidity of 75–78%.
Name and location of the manufacturer (production facilities)	+	+	+	+
Additional marks (statements)	'Recommended for preschool and school age children' 'GMO FREE'	'Recommended by the Ministry of Health of Ukraine for preschool and school age children (from 3 years old)' WITHOUT SOYA 'Gluten Free' 'From selected meats' 'Beef 50% / Pork 40%"	'GMO FREE'	'Recommended for preschool (from 3 years old) and school age children' 'GMO FREE'

Thus, we see that the current regulatory documentation is now inconsistent. On the one hand it regulates the technical requirements for the production of sausages, and on the other hand it sets the rules of labeling. In addition, in article 47 of 'The order of organization of food in educational institutions and children's health and recreation institutions' (CMU, 2021) states 'In educational institutions, health facilities and recreation is prohibited to order and use/sell technologically processed meat and fish products'.

To sum up the analysis of current and project documentation, as well as data on the possible negative impact of certain ingredients, we consider it is unacceptable to use in the text of DSTU 4436:2005 (DSSU, 2006) the indication 'Sausages recommended by the Ministry of Health for baby diet', therefore it is necessary to update the national standard.

At the next stage of research, a visual and sensory analysis of prototypes has been conducted. Deviations in the appearance of experimental samples of sausages 'Dytiachi' of the premium grade were not detected.

After the external examination, sausages were cut and their consistency, color and appearance of minced meat, smell and taste were determined. It was found that the consistency of sausage bars 'sample 1' is elastic, but tender and juicy, minced meat on the cut surface is light pink and homogeneous, without grey spots and cavities; the smell and taste are pleasant, with a milky aftertaste, moderately spicy and moderately salty. The broth was transparent, slightly pink, with a few small drops of fat during the cooking of samples.

The study of organoleptic characteristics of sausages 'sample 2' found that the sausages had an elastic and tender texture, were juicy, the color of the cut surface is pale pink, homogeneous; the smell is specific and meaty, the taste is moderately salty, there is a moderate amount of spices and milky taste. During the cooking test, the broth turned out to be transparent, pale pink, fragrant, with single drops of fat on the surface.

The organoleptic of the sausages 'sample 3' revealed an elastic but friable consistency, pinkish-grey color of the sausage stuffing; pronounced characteristic smell, salty, with a moderate amount of spices, taste. In the way of cooking revealed that the broth is cloudy, pale pink with drops of fat on the surface.

The study of sausages 'sample 4' found that the consistency of the sausage is elastic, tender and juicy, and the minced meat in the cut is light pink and homogeneous; the smell is delicate and specific, a moderate amount of spices and salt is felt. The broth was pale pink, transparent, fragrant, with a few drops of fat on the surface.

Generalized results of sensory evaluation of sausages are shown in Fig. 1. The figure shows that the highest number of points — 29.6 — scored sausages 'sample 2', sausage 'sample 1' and 'sample 4' also received quite high scores — 29.2 and 28.9 points, respectively. Instead, sausages 'sample 3' were significantly resigned to all studied indicators of the other studied samples and received the lowest overall score — 26.7. However, it is important to note that despite the significant difference in the number of scores obtained by the results of sensory evaluation, all samples meet the requirements of the national standard for organoleptic parameters.

According to the results of determining the mass fraction of the main components of the chemical composition of the experimental samples of sausages (Table 2), it was found that the mass fraction of protein ranged from 11.97 ± 0.11 to 13.7 ± 0.26%, mass fraction of fat — from 16.66 ± 0.27 to 17.01 ± 0.31%, mass fraction of moisture — from 64.6 ± 0.23 to 66.8 ± 0.44%, which meets the requirements of DSTU 4436:2005 (DSSU, 2006) depending on the manufacturer. Regarding the salt content, in sausages 'sample 1' (1.98 ± 0.01%), 'sample 2' (1.96 ± 0.08%) and 'sample 4' (1.99 ± 0.1%) its mass fraction met the requirements of the national standard, however in the product 'sample 2' the figure was 2.11 ± 0.14%, which is 0.11% higher than the norm regulated by DSTU 4436:2005 (DSSU, 2006).

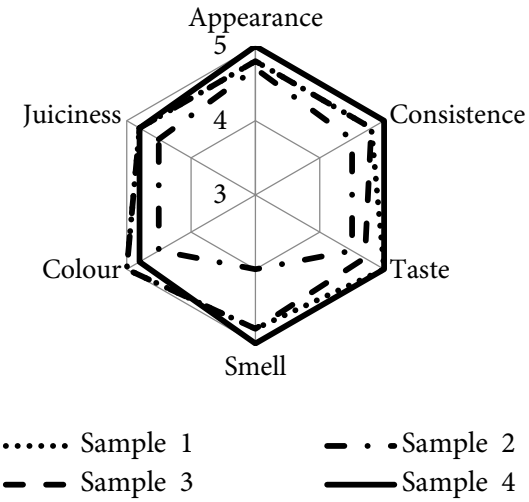

Figure 1. The results of sensory evaluation of sausages 'Dytiachi' premium grade from different manufacturers

Table 2 — Indicators of the chemical composition of sausages 'Dytiachi' premium grade, M ± m, n = 5

Name of indicators	DSTU requirements	Tested samples			
		Sample 1	Sample 2	Sample 3	Sample 4
Mass fraction of protein, not more than, %	12.0	12.1 ± 0.13	13.17 ± 0.26	11.97 ± 0,11	11.9 ± 0.11
Mass fraction of fat, not more than, %	17.0	16.77 ± 0.54	16.66 ± 0.27	17.01 ± 0.31	16.87 ± 0.22
Mass fraction of moisture, not more than, %	68.0	65.9 ± 0.48	64.96 ± 0.23	66.04 ± 0.51	66.8 ± 0.44
Mass fraction of kitchen salt, not more than, %	2.0	1.98 ± 0.01	1.96 ± 0.08	2.11 ± 0.1	1.99 ± 0.11

According to the results of microbiological studies (Table 3), it was found that all experimental samples of sausages meet the requirements of DSTU 4436:2005 (DSSU, 2006). The content of MAFAnM is in the range

from $(1.7 \pm 0.13) \times 10^2$ to $(2.6 \pm 0.19) \times 10^2$ CFU/cm^3. BGCP (coliform bacteria), sulfite-reducing clostridia and staphylococci in 1 g and salmonella and listeria in 25 g of the product were not detected.

According to the results of the study of the content of ^{137}Cs and ^{90}Sr radionuclides (Table 4), it was established that their content did not exceed the levels regulated by the national standard.

Table 3 — Microbiological indicators of sausages 'Dytiachi' premium grade, M ± m, n = 5

Name of indicators	DSTU requir-ements	Tested samples X×10^2 CFU/cm^3			
		Sample 1	Sample 2	Sample 3	Sample 4
Quantity of MAFAnM, CFU/cm^3	not more 1,0×10^3	1.8 ± 0.02	1.7 ± 0.13	2.6 ± 0.19	2.1 ± 0.21

Table 4 — The content of radionuclides in sausages 'Dytiachi' premium grade, M ± m, n = 5

Name of indicators	DSTU requir-ements	Tested samples, Bq/kg			
		Sample 1	Sample 2	Sample 3	Sample 4
^{137}Cs	200	5.31 ±0.21	6.33 ±0.19	4.53 ±0.13	5.44 ±0.61
^{90}Sr	20	0.53 ±0.14	0.64 ±0.22	0.46 ±0.37	0.53 ±0.21

Thus, the analysis of the results of research on the quality of sausages of the premium grade 'Dytiachi' found that the test products 'sample 3' does not meet the requirements of the national standard for the mass fraction of salt, which is unacceptable, especially for products approved to be used by children.

Conclusions. Labeling of packaging of tested samples of sausages 'Dytiachi' contains basic production and consumer information in accordance with paragraph 1 of article 6 of the Law of Ukraine 'About information for consumers about food products' (VRU, 2019).

However, none of the samples contains information on the mass content of saturated fats and salt, moreover the carbohydrate content is indicated only on the label of sample No. 2, which violates the requirements of article 23 of this law.

When labeling test samples No. 1, 2, and 4, manufacturers use the statement 'Recommended for diet of preschool children (from 3 years) and school age', which is consistent with the text of DSTU 4436:2005 'Boiled sausages, frankfurters, sardellas, meat loaves. General specifications' (DSSU, 2006), but contradicts the 'Requirements for claims on the nutritional value of foods and claims on the health benefits of foods' (MHU, 2020).

According to the results of the study of chemical composition, it was found that in sample No. 2 the mass fraction of table salt was 2.11 ± 0.14%, which exceeds the content regulated by the state standard by 0.11%.

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Part 3. Biosafety