

## VIROPHORY OF THE PIG'S MICROFLORA AS A PHENOMENON OF THE SOME PORCINE VIRAL INFECTIONS PERENNISATION

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**Summary.** The problem of the perennisation porcine viral infections/rooting their agents is far from clear understanding. The article proposes a mechanism for the rooting of porcine viral infections through the interaction of viruses with the porcine microbiome. This provision is stood on results of retrospectively examine the relationship between bacterial virophoria of the primary microbiological cultures and the enzootic foci formation on the model of two enzootic foci of porcine respiratory disease complex (PRDC) with including of agents of the Aujeszky's disease (AD) and porcine circovirus infection (PCVI). There was studied 183 samples of primary bacterial cultures (BC) from samples of clinical and pathological materials taken from pigs during the outbreaks/'PRDC red phases' ('exposed pigs') and in 'PRDC green phases' ('non-exposed pigs'). There AD agent virophoria detected in 29 bacterial samples (70.7%, BC from nasal mucus, semen, liver and spleen) and PCVI agent — in 22 samples (59.5%, BC from nasal and vaginal mucus and lungs) were recorded in group of 'exposed pigs'. But there only 5 from 142 bacterial samples (3.5%, BC exclusively in semen samples) was recorded in group of 'unexposed pigs' in both holdings as virophoric for AD agent and two from 20 samples as virophoric for PCV-2 (11.1%, BC from lung and vaginal mucus samples). In according EvansCounty calculation (Epi Info for Windows v. 7.1.5) these date did next significance odds ratios (OR) and risk ration (RR) indexes ( $P = 99.99\%$ ):  $16.12 \leq OR = 43.17 \leq 134.05$  and  $3.13 \leq RR = 4.25 \leq 5.77$ , respectively. In addition, there BC from 11 nasal swabs of pigs with clinical signs of PRDC in back-yard holdings after the lifting of the quarantine for African swine fever (ASF) was examined in PCR on ASF. The 5 BC samples revealed as virophoric. To further develop of the Kharkiv doctrine of the associated infections epizootiology the concept of switching the epidemic process into an endemic one in piggery through the direct interaction of viruses with the pig microbiome is proposed

**Keywords:** Aujeszky's disease, porcine circoviral disease, African swine fever

**Introduction.** Over the past 50 years, the commercial swine industry in Ukraine has controlled a number of viral infections that were considered particularly dangerous before the advent of appropriate vaccines. The vaccines used in Ukraine are guaranteed to protect vaccinated pigs from death and severe clinical manifestations of these infections. However, no vaccine can prevent all field variants of the respective pathogens from colonizing vaccinated pigs (Farrington, 2003; Rose and Andraud, 2017).

Therefore, the vaccination itself did not stop the circulation of the pathogens of these infections. Moreover, almost all of them have taken root in Ukraine as factorial diseases or syndrome complexes in various enzootic foci. In industrial pig production, this is accompanied by the clinical manifestation of various syndromes of associated viral and bacterial infections (Delon, 2022).

A typical example of this pattern in the world pig industry (and Ukraine is no exception), since the early 1980s and to date, is the polymicrobial syndrome complex of severe respiratory disease in pigs/porcine respiratory disease complex (PRDC). Its etiological components at different periods were associated with pathogenic and opportunistic pathogens with tropism to the tissues of the respiratory system of pigs (mycoplasmas, pneumococci, *Bordetella* spp., *Pasteurella* spp., certain species of micromycetes, etc.). However, as triggers of

this syndrome complex serve usually porcine viruses with strong immunosuppressive properties and pulmonary tropism (Zimmerman et al., 2019).

Until the mid-1990s, such a virus was undoubtedly the Aujeszky's disease (AD) agent (Fuentes and Pijoan, 1987; Sakano et al., 1993).

Until in Ukraine, it periodically sometime 'replace' in the etiologic microflora of the PRDC by swine influenza virus (SIV) and/or respiratory coronavirus (RCV) (Hill et al., 1989; Wentworth et al., 1994).

At the beginning of the millennium, under AD scenario, i.e. after the massive use of appropriate vaccines, porcine reproductive and respiratory syndrome (PRRS) and porcine circovirus infection (PCVI) agents came to the forefront of the etiology of PRDC (Allan et al., 2000; Kim, Chung and Chae, 2003; Opriessnig, Giménez-Lirola and Halbur, 2011).

Today, these viruses are actively involved in the formation of enzootic foci of associated forms of African swine fever (ASF) (Assavacheep and Thanawongnuwech, 2022; Buzun et al., 2023).

The causal component of PRDC has always been and remains gross violations of intensive pig production biosafety requirements, the feed's low nutritional quality, and technological stress (Yu et al., 2021; Ramos, Sibila and Neira, 2023). Mentioned data can indicate that many different viruses that were exotic for Ukrainian piggery, now have taken root in the pig industry under similar

scenarios of transforming viral mono infections into enzootic foci of associated syndrome infections such as PRDC. This gives grounds to assume that the mechanism of their establishment is universal.

We assumed that this mechanism might be related to the phenomenon of bacterial virophoria, which was first described in the 1930s by soviet microbiologists under the leadership of Prof. Zilber (1956). Subsequently, this phenomenon was most actively studied at Ukrainian Scientific Research Institute of Experimental Veterinary (now the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'). In the 1990s, Kharkiv scientists considered it as a sign of the associative activity of microorganisms in the paradigms of the Kharkiv Epizootiological School (Apatenko, 2006; Buzun and Apatenko, 2003) and tried to apply it in the biotechnology of viral drugs (Fuks et al., 1995). We regard the prospect of study of bacterial virophoria as an driving forces of the epizootic process as an next step of development of the Kharkiv Epizootiological School for associated infections. Therefore, our current study aimed to retrospectively investigate the relationship between bacterial virophoria and the formation and functioning of enzootic foci of emergent (at some time ago) viral infections. The results of the epizootic analysis of monitoring data conducted in 2020–2022 in two enzootic focuses of the PRDC with the participation of a consortium of AD and PCVI agents with 'respiratory' pig bacteria are presented below.

#### **Materials and methods. Epizootiological background.**

During the monitoring period, the two target pig farms in the eastern and central regions of Ukraine were characterized as stationary for PRDC with the PCVI and AD etiology. In particular, the pig complex of the cereal-based agri-food enterprise group 'Vilne-2002' (established in 2002, latitude 48.74459, longitude 35.292854) is a typical commercial farm of the intensive type with a full pig production cycle, with a total number of animals (Large White, Landrace) of 27–29 thousand pigs, of which 2.3–2.5 thousand pigs are breeding animals. In 2018–2022, according to the results of laboratory tests, the following infections were recorded at the clinical level (sporadic cases and periodic outbreaks in certain technological groups): actinobacillar pleuropneumonia (APP), pasteurellosis and pneumococcosis, PCV-2, AD; at the subclinical level (seropositivity and/or carriage of the pathogen by the results of semen, nasal and vaginal swabs) — the same infections and additionally — PRRS (after the use of commercial virus vaccine against PRRS on virus carriers of the AD agent). In healthy pigs, during the whole monitoring period, seropositivity for AD ranged from 45% to 70%, and for PCVI — from 15% to 90%; as well as carriage of AD and PCVI pathogens by the results of analysis of semen, parenchymal organs, nasal and vaginal swabs (Tables 2 and 3). The waves of exacerbation of infections from the PRDC spectrum were extinguished by the owners by the massive use of commercial broad-spectrum antibiotics (Tylosin-200, Vitadox, Ceftriaxone-Darnitsa, Ofloxacin-Darnitsa, Efikur)

and the experimental composite 'NanoViroSan' based on official antiviral drugs (Buzun, Kolchyk and Paliy, 2024) with virostatic and immunostimulating activity — followed by vaccination of the recovered pigs with an emulsion vaccine of local strains of PCVI and APP pathogens, as well as labeled vaccines 'Suimun ADIVAC' against AD from BioTestLab and 'Ingelvac ZircoFLEX' against PCVI from Ingenasa.

AMO-K LLC is a breeding reproducer for the Large White pigs (launched in 2005) (founded based on commercial pig production in 2001; latitude 49.804049, longitude 30.580705). In 2018–2022, according to the results of laboratory tests, the following infections were registered at the clinical level: pasteurellosis and neisseriosis (pneumococcal and gonococcal infection), circovirus infection (both with the clinic of 'acquired piglet wasting' and in the form of 'porcine renal and skin syndrome'), Aujeszky's disease (splenomegaly with hepatonecrosis at autopsy); at the subclinical level (seropositivity for AD from 7% to 50%, and for PCVI — from 80% to 95%; also carriage of AD and PCVI pathogens by the results of analysis of parenchymal organs and nasal flushes (Tables 2 and 3). Tables 2 and 3 are the same infections, and respiratory coronavirus infection is also present. To suppress PRDC outbreaks, pig farmers used commercial antimicrobials (Florpan C, Enrolen 10%, Megasil KLA, Bicillin 3 powder) and vaccines (Porcylis Begonia from MSD Animal Health Ukraine against AD and Flexcombo from Boehringer Ingelheim against PCVI and porcine enzootic pneumonia) according to their manufacturers' recommendations.

All vaccines on both farms were used according to the decisions of the pig farm managers without systematic detection and culling of seropositive pigs in the breeding herd, as recommended by the WHO for labeled vaccines.

In addition to these pig farms, a clinical examination of sick pigs (n = 37) was conducted in three private households in Dnipropetrovsk Region, which revealed signs of chronic respiratory disorders characteristic of PRDS. Taking into account the current situation in the pig industry regarding African swine fever (ASF), it was decided, along with the relevant notification of the regional inspection of the State Service of Ukraine on Food Safety and Consumer Protection, to conduct a study not only for PRDS but also to examine the nasal mucus of chronically sick pigs for the presence of ASF agent' DNA.

**Sampling and sample preparation.** The samples were taken from parenchymal organs (spleen, kidneys, liver, lungs, tonsils, lymph nodes) from dead and culled pigs (n = 83), and samples of nasal (n = 96) and vaginal mucus (n = 24) on swabs, as well as portions of boar ejaculate (n = 33), selected by the veterinary service of the pig farms 'Vilne-2002' and 'AMO-K' in the period 2018–2022 and delivered to the NSC 'IECVM' according to the current requirements (SDVMMAU, 1997; Shabbir et al., 2013) together with the data from the epizootic survey.

These samples, together with primary bacteriological cultures (more than  $n = 300$ ), were stored in a bacteriological preservative medium containing glycerol at a temperature of  $-40^{\circ}\text{C}$  after diagnostic tests. In 2023–2024, 183 of these bacteriological cultures were tested for virophoria in 2–3 replicates each. In total, 94 diagnostic samples and 41 bacteriological culture samples from these samples were examined, which, according to the epizootic survey, represent the period of clinical aggravation of PRDS on farms (i. e. the period of the 'red phase' of biosafety of pigs in the enzootic PRDS focus); and 142 diagnostic samples and 142 samples of cultures from them, which, according to the survey data, were collected on these farms in the periods from 3 to 7 weeks after the stabilization/decrease of the epizootic PRDS situation ('green phase' of biosafety of PRDS) (Stegniy et al., 2010).

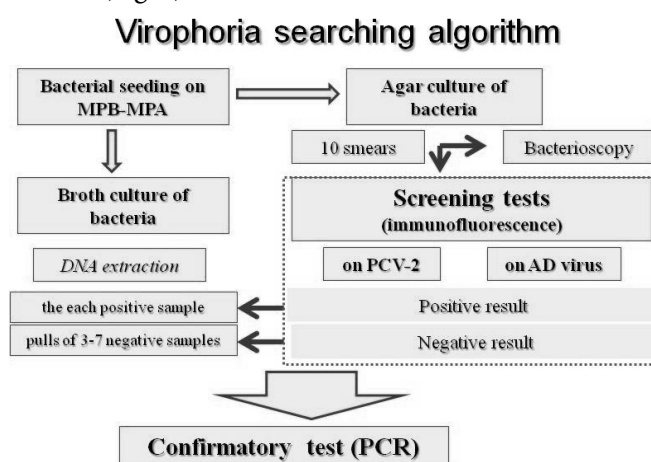
In addition to these industrial pig farms, sampling was carried out by the regional veterinary service in three private pig farms during the acute phase of PRDS: nasal mucus ( $n = 19$ ) and blood ( $n = 9$ ) and pathologic material (spleen, lungs) from pigs culled due to disease ( $n = 6$ ) samples were collected. For preliminary estimation there lateral immunodiagnostic test was used. The blood samples were directed to the Sumy Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection on agents of the PRDS pathogens (PCV-2, AD, influenza A, mycoplasmosis, *Pasteurella*-like bacteria). Also to this laboratory for testing on ASF were directed the bacterial cultures from nasal mucus samples which prepared in 'Optim-Vet' Ltd as described below (see *Study of bacterial virophoria*).

**Laboratory and diagnostic studies** in these farms were carried out in the period 2018–2022 under the current standard operating procedures of the NSC 'IECVM': SOP No. 4/17.12.2013 on the identification of swine viruses using an immunoperoxidase monolayer test and ELISA; SOP No. 5/17.12.2013 on the isolation and identification of pathogens of swine viral diseases; and SOP No. 2.1-2017 'Isolation, identification, maintenance and storage of museum cultures of infectious agents of pigs', developed taking into account the requirements of the WOAHA Terrestrial Manual (WOAHA, 2024).

The identification of pathogens was performed by immunoperoxidase and/or immunofluorescence methods (screening tests) accompanied by PCR (confirmatory test). Antibodies against these pathogens in blood samples were detected in the indirect hemagglutination test (screening test) with confirmation of the specificity of the reaction in ELISA (confirmatory test). In bacteriological studies, according to the specified SOPs of the NSC 'IECVM', traditional methods of isolation and identification of bacteria and micromycetes were used (including primary bacterial cultures on meat-peptone broth-agar (MPB-MPA) and subsequent growing on differential bacterial media, staining, and microscopy of bacterial smears, etc.) at the certified workplaces of the NSC 'IECVM'.

For field trials there lateral immunodiagnostic test on base of test strips ASF-Ag (<https://www.alltests.com.cn/Home/ProductInfo/617>) and Porcine Pseudorabies Virus gE (PRV-gE) Ab Rapid Test (<https://www.alltests.com.cn/Home/ProductInfo/278>) manufactured by Vechek (China) was used by 'Optim-Vet' Ltd personals.

**Study of bacterial virophoria.** The study of bacterial virophoria concerning PCVI and AD agents from samples of biological material taken from pigs in PRDS enzootic foci was performed according to the following scheme (Fig. 1).



**Figure 1.** The scheme of the bacterial virophoria study.

To determine the virophoric bacteria, 10 standard swabs were made from a suspension of agar colonies with a concentration of 0.5 IU McFarland on slides, which, after fixation, were stained by Gram and Romanowsky–Giemsa, also by fluorescent antibody (FA) and immunoperoxidase monolayer (IPM) methods. To detect the antigens of PCVI and AD viruses in FA (screening test; IPM as additional experimental test), the swabs of the cultures were fixed with 96% ethanol. The specificity of this test was confirmed by examining the broth culture of each positive sample of the cultures, and the specificity of the negative reaction was confirmed for a pool of broth cultures from three to seven negative samples. For IPM some broth culture samples were precipitated into bacterial precipitate by low-speed centrifugation and resuspended in a lincomycin solution (10 U/ml) in a sterile 0.15 M sodium acetate buffer with pH 4.5. For a virus elution these suspensions exposure at  $28\text{--}30^{\circ}\text{C}$  (water bath) for 45 min, each sample was filtered through a  $40\text{ }\mu\text{m}$  pore Bond Elut SI cartridge. After alkalization to pH 7.2–7.4 with 0.1% enrofloxacin solution the filtrates were inoculated in 5<sup>th</sup>–7<sup>th</sup>-day-monolayer cultures of primary porcine alveolar macrophages (PAM) on glass slides in cultural tubes. For the detection of AD and PCV-2 viruses, the monolayer of infected (experimental) and uninfected (control) PAM was fixed with 96% ethanol (1 h; room temperature, RT) 3–5 days after inoculation. The endogenous peroxidase of PAM cultures was inactivated with 0.01% hydrogen peroxide solution (20–25 min at a temperature of  $37^{\circ}\text{C}$ ).

Identification of isolated viruses was applied indirect immunoperoxidase reaction on base of appropriate virus-specific, control sera and conjugate (protein A-peroxidase) taken in work dilutions from certified diagnostic; detection of peroxidase — with ortho-dianisidine (Buzun, 1983; EURL-ASF, 2013). As a confirmatory test, the PCR were used: in according to the relevant protocols recommended for the identification of PCV-2 and AD viruses (Neumann, Ramirez and Schwartz, 2020; WOA, 2024). For this purpose, DNA was extracted from broth culture samples (Fig. 1), with a

concentration of 0.5 IU McFarland, using the ‘TIANamp Virus DNA/RNA Kit’ (Tiangen, Beijing, China), according to the manufacturer’s guidelines. Primers ‘PCV2-Q-F/R’, ‘PRV-Q-F/R’, and ‘ASFV F/R’, which WOA recommends for diagnostic studies, were used to assess the levels of PCV-2, AD, and ASF agents DNA accumulation in bacterial cultures (Table 1). RT-PCR tests for PCV-2 were performed at the NSC ‘IECVM’, and with DNA samples for AD and ASF — on request, in the certified PCR laboratories of Sumy Region, according to the protocols recommended by WOA (2024).

**Table 1** — Sequence of primers used

Primer	Sequence (5'→3')	References
PCV2 Cap-Q-F	TGTAGTATTCAAAGGGGCACAGAGC	Shen et al., 2008
PCV2 Cap-Q-R	CGGATATACTATCAAGAAAACCCAC	
PRV gD-Q-F	GGTTCAACGAGGGGCCAGTACCG	Peng et al., 2016
PRV gD-Q-R	GCGTCAGGAATCGCATCACGT	
ASFV Fwd	CTGCTCATGGTATCAATCTTATCGA	King et al., 2003
ASFV Rev	GATACCACAAGATCRGCCGT	

The *epizootiological analysis* was conducted in the EvansCounty format using the methods of observational and cohort studies, using the CDC USA software — Epi Info for Windows v. 7.1.5 according to the developer’s requirements (<https://www.cdc.gov/epiinfo>).

**Results. Collection and clustering of monitoring data in comparison with bacterial cultures virophoria data.** The laboratory and diagnostic data obtained in 2019–2022 were initially distributed by biosecurity phases of both target pig farms enzootic for PRDC with the participation of PCVI and AD agents: Cluster I of monitoring data obtained from the pig farms ‘Vilne-2002’ and ‘AMO-K’ in the ‘red phase of PRDS biosecurity’ (total n = 94 field samples) and Cluster II from the same farms, but in the ‘green phase of biosecurity’ (total n = 142 field samples). After the study in 2023–2024 of bacterial cultures made from the preserved part of the same samples, the diagnostic data were supplemented with experimental data on the virophoria of the bacteria of the corresponding cultures: in Cluster I, bacterial cultures from 41 field samples, and in Cluster II, from 142 field samples. The monitoring results obtained for both samples (n = 419 samples in total) are summarized in Tables 2 and 3. It was found that the procedure of freezing-thawing of bacterial cultures contributes to a more confident detection of both circovirus and AD virus in PCR and by viral isolation in cell culture, but negatively affects the efficiency of their detection in bacterial swabs by fluorescent antibody. Culture bacteria from samples of biological material exclusively positive for AD and/or PCVI agents were positive for virophoria, respectively.

The virophoria of the AD pathogen on both farms was studied in 183 samples of bacterial cultures (41 samples from the periods of the ‘red phase of PRDC biosafety’ and 142 samples from the ‘green phase’): they were fully tested for AD, and 57 samples were tested for PCVI.

Virophoria of AD and PCVI agents was recorded only in primary bacterial cultures (n = 58): passage bacteria were free of these viruses (Tables 2 and 3). In total, from 41 samples of the cultures collected during the ‘red phase of PRDC biosafety’ (Table 2), AD pathogen virophoria was detected in 29 samples (70.7% in cultures from nasal mucus, semen, liver and spleen), and PCVI agent in 22 samples (59.5% in cultures from nasal and vaginal mucus and lungs).

At the same time, from 142 samples of ‘green phase’ of PRDC biosafety samples (Table 3), AD pathogen virophoria was recorded in 5 from 142 samples (3.5% exclusively in semen samples), and PCVI agent in two from 20 samples (11.1% in lung and vaginal mucus samples).

In the course of these studies, the virophoria of AD and PCVI pathogens was recorded in polymicrobial consortia that included the following microorganism species: *Pasteurella multocida*, *Mannheimia haemolytica*, *Actinobacillus pleuropneumonia*, *Haemophilus parasuis*, *Mycoplasma hyopneumonia*, *Neisseria pneumonia*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Streptococcus haemolyticus* and/or *Bacillus cereus*, usually (especially in the inter-epizootic period) in association with *Candida albicans* or other micromycetes.

In particular, a clear difference in the microbiological profile of the studied PRDS foci was observed in different periods of their activity (research is ongoing). Preliminary data have also been obtained on certain selectivity in the interaction of viruses with bacteria. Research in these areas is ongoing.

**Assessment of the available reliability for the data.** According to the Epi Info 7 CDC Users Guide, before conducting an analysis in the EvansCounty format, it is first necessary to determine whether the targeted samples of epidemiological data are capable of providing the required level of confidence in the analytical conclusions.

**Table 2** — Summary data for 2019–2024 from the testing of samples (n = 94) from stationary foci of PCVI and AD mixed infection in the ‘red phase’ \* of PRDC biosafety of the pig farms (n = 2)

Sample characterization			Number of positive/negative test results					
Pig farms	Type	Number	Diagnostic			Virophoria of bacteria cultures **		
			PCVI	AD	Total	PCV-2	ADV	Total
‘Vilne-2002’	nasal swabs	24	15/1	15/9	24	14/1	13/3	16
	vaginal swabs	6	6/0	0/6	6	4/0	0/3	3
	sperm	6	3/3	5/2	7	0/3	4/0	4
	parenchymal organs	33	10/24	14/18	32	3/4	3/2	5
‘AMO-K’	nasal swabs	16	0/16	14/2	16	0/4	8/1	9
	parenchymal organs	9	7/9	8/1	9	1/3	1/3	4
Total samples		94	94			37	41	41
of these, positive/negative		—	41/53	56/38	—	22/15	29/12	—

Notes: \* — the ‘red phase of biosafety’ in industrial pig farms is a period of extreme exacerbation of the epizootic situation on the farm; in this case, in terms of mass clinical manifestations of PRDS syndrome; \*\* — no bacterial cultures were made from 16 field samples (not preserved); 4 from 41 cultures were not tested for PCV-2 virophoria.

**Table 3** — Summary data for 2019–2024 from the study of samples (n = 142) from stationary foci of PCVI and AD mixed infection in the ‘green phase of PRDC biosafety’ \* of pig farms (n = 2)

Sample characterization			Number of positive/negative test results					
Pig farms	Type	Number	Diagnostic			Virophoria of bacteria cultures **		
			PCVI	AD	Total	PCV-2	ADV	Total
‘Vilne-2002’	nasal swabs	35	0/23	3/35	38	0/5	0/35	35
	vaginal swabs	18	9/18	0/8	8	1/4	0/18	18
	sperm	27	5/27	13/27	40	0/4	5/27	32
	parenchymal organs	34	9/34	0/28	28	1/3	0/34	34
‘AMO-K’	nasal swabs	21	0/17	5/21	26	0/2	0/21	21
	parenchymal organs	7	—	0/2	2	—	0/2	2
Total samples		94	142			20	142	142
of these, positive/negative		—	23/119	21/121	—	2/18	5/137	—

Notes: \* — the ‘green phase of biosecurity’ in industrial pig production is a period of safe epizootic situation in the farm; in this case, it occurred within 3–5 weeks from the beginning of scientifically based anti-epizootic measures to eliminate the PRDS outbreak; \*\* — 122 cultures were not tested for PCV-2 virophoria.

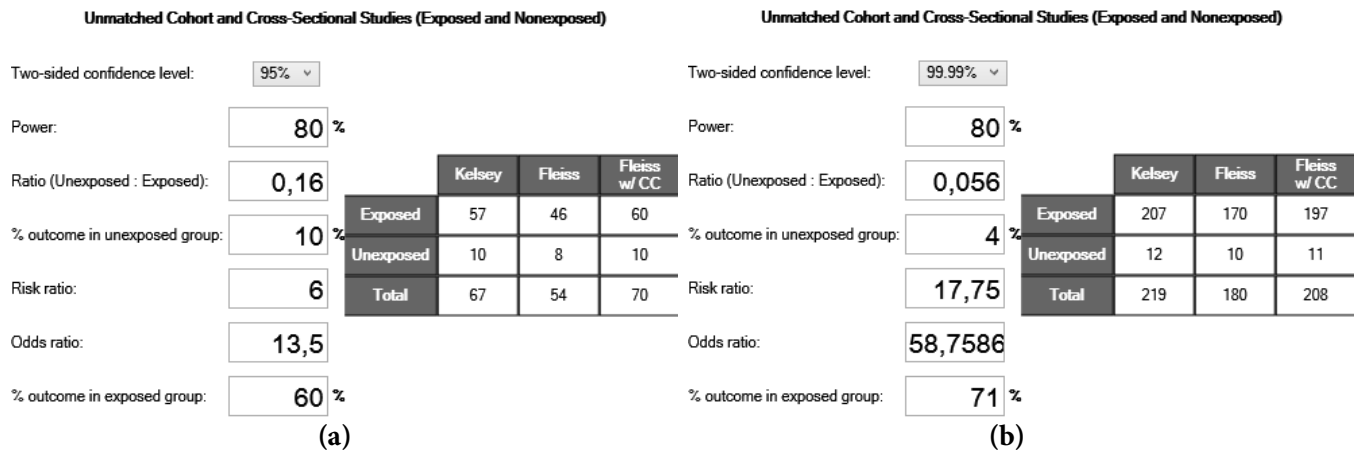
According to the summary data in Tables 2 and 3, we have a total sampling of two farms at two biosafety levels for PRDC (as probable positive and negative virophoria results) for PCV-2 virophoria in the total amount of n = 57, and for AD — in the amount of n = 183 (i. e. positive/‘exposed’ and negative/‘non-exposed’ samples from these two farms). At the same time, the virulence of the PCVI agent (‘probable outcome’) was set at 60% (n = 22 from 37) at the ‘red PRDC biosafety level’, and at the ‘green level’ — 10% of samples (n = 2 from 20). That is, it was experimentally established that the percentage of bacterial virophoria in samples from pigs obtained during the PRDC outbreaks for PCVI is 60%, and after the PRDC outbreaks is over — 10%: this gives a value of ratio (unexposed:exposed) = 0.16. Therefore, with a data set of n = 57 samples (PCV-2), we can count on a statistical confidence level (two-sided confidence level) of the future conclusions of the epizootic analysis at the level of 95% (Fig. 2a).

At the of both pig farms ‘red level of biosafety’, the AD pathogen virophoria was found in 71% of bacterial cultures (n = 29 from 41), and at the ‘green level’ — in

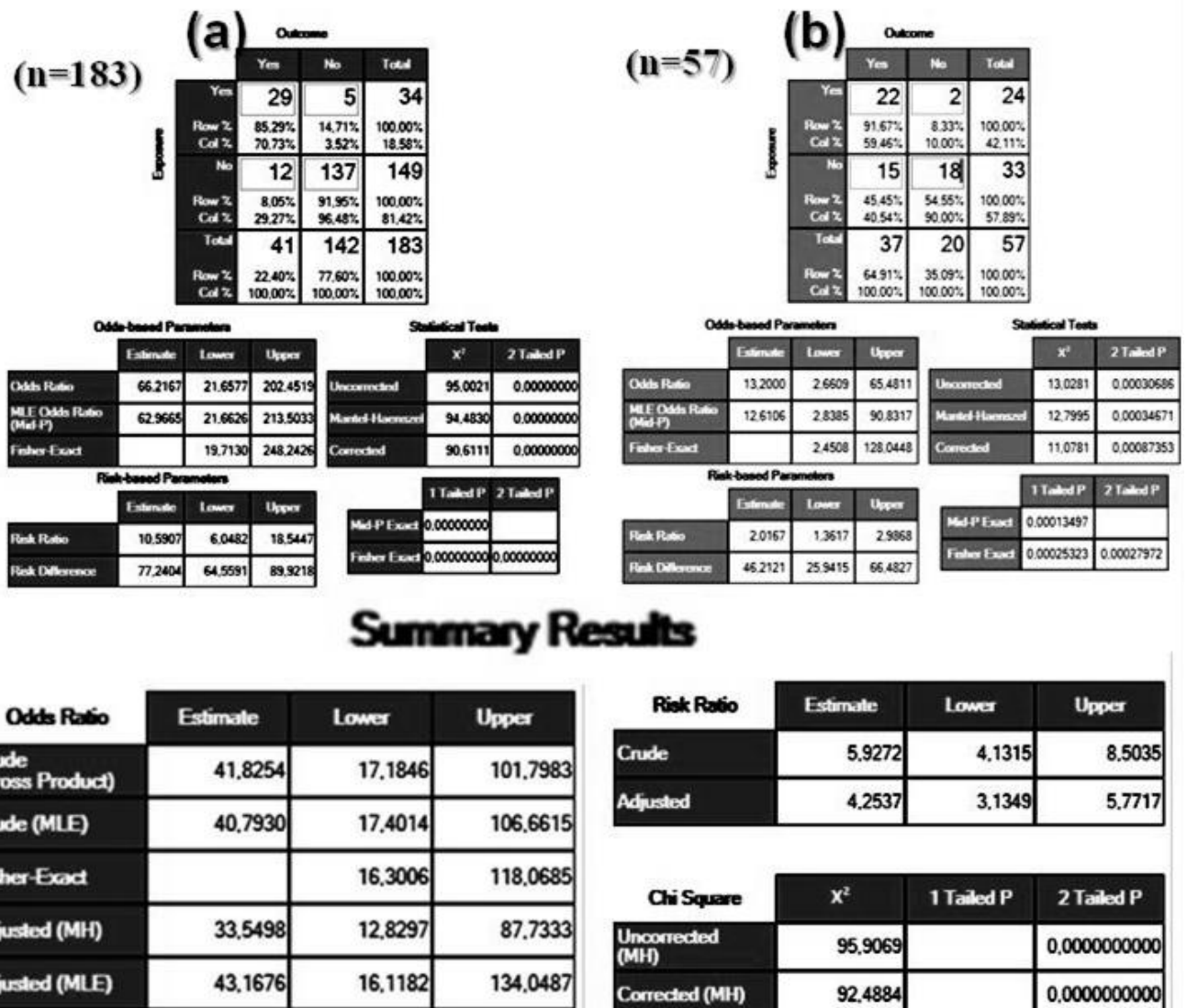
4% of bacterial cultures (almost exclusively from semen, n = 5 from 137). As seen in Fig. 2b, this gives a value of the ratio (unexposed:exposed) = 0.056. Thus, with a data set of n = 183 samples, we can count on the highest statistical confidence in the future conclusions of the epizootic analysis (two-sided confidence level) at 99.99%.

**Evaluation of the bacterial virophoria impact on the enzootic process of PRDC involving PCVI and AD agents.** The results of calculating the odds ratio (OR index) and the risk level (RR index) of activation of PRDS enzootic foci through the mechanism of bacterial virophoria are presented in Fig. 3.

A total of 183 samples were tested for AD, of which 57 were tested for PCVI. For the AD virus, calculations in the EvansCounty format (Fig. 3a) on the sets of samples (total n = 183) from both pig farms collected in the ‘red’ (n = 34) and ‘green’ biosecurity phases (n = 149) and both positive and negative for AD (n = 41 and n = 142, respectively) at the P < 0.000 level, it is shown that the odds ratio index of the target phenomenon in the exposed groups (in the horizontal bars of the upper part of Fig. 3a) is OR = 66.2.



**Figure 2.** Determination of the two-sided confidence level of the data obtained on the virophoria of PCVI and AD pathogens and bacterial cultures of samples from stationary PRDS foci (n = 2)



**Figure 3.** Results of calculation of the OR and RR indices of the probability of participation of bacterial virophoria in relation to PCV-2 (a) and AD virus (b) in the activation of PRDC enzootic foci.

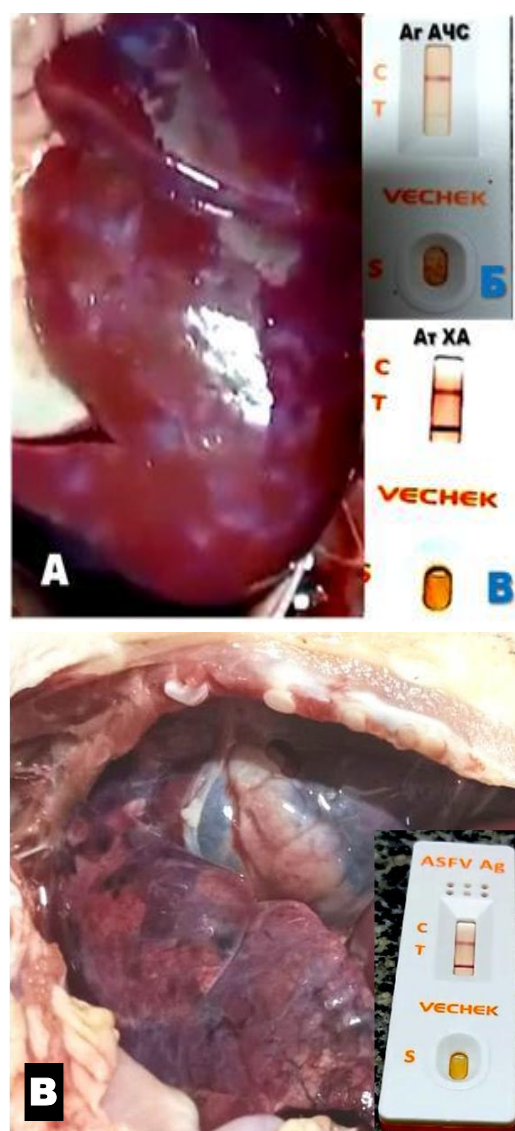
The risk ratio index of PRDC exacerbation between the groups of positive and negative samples (in the vertical columns of the upper part of Fig. 3a) is  $RR = 10.6$ .

For PCV-2 (Fig. 3b), on the sets of samples (total  $n = 57$ ) from both pig farms collected during the periods of PRDC exacerbation ( $n = 24$ ) and 'epizootic lull' (i. e. 3–5 weeks after measures to eliminate periodic outbreaks,  $n = 33$ ) and simultaneously positive and negative for PCV-2 ( $n = 37$  and  $n = 20$ , respectively) at the level of  $P \leq 0.001$  showed that the odds ratio index for the target event in the exposed groups (horizontal bars in the upper part of the table) was  $OR = 13.2$ , and the hazard ratio index for PRDC exacerbation based on the results of the EvansCounty calculation of the sets of positive and negative samples (vertical columns in the upper part of Fig. 3b) was  $RR = 2.02$ .

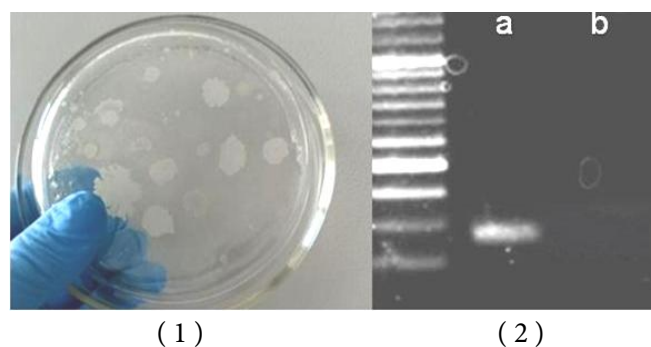
The data in Fig. 3 in part 'Summary results' indicate a strong causal link between bacterial virophoria as such and the worsening of the situation in both studied enzootic foci of PRDC according to the parameters of PCVI and AD mixed infection. This is convincingly evidenced at the level of statistical significance  $p \leq 0.000$  ( $n = 240$ ) by the powerful level of  $OR$  ( $16.12 \leq OR = 43.17 \leq 134.05$ ) and  $RR$  ( $3.13 \leq RR = 4.25 \leq 5.77$ ) indices.

**Study of ASF agent virophoria on bacteria from pig nasal mucus cultures.** When studying the etiology of PRDC in pigs ( $n = 19$ ) from three households in Dnipropetrovsk Region after the lifting of the quarantine against ASF, the following was established. All blood samples ( $n = 9$ ) were positive for AD antibody according to the results of laboratory tests in ELISA. At the same time, antibodies against the AD virus were detected in the field test even in the ascites fluid and pulmonary transudate of six pigs culled due to severe clinical and post-sectional signs of PRDC (Fig. 4). In two of them, whose lungs and liver were most affected, the pulmonary transudate according to the results of the field test contained the antigen of the ASF virus — as low (Fig. 4A) and strong positive (Fig. 4B) on glycoprotein gp 72 of its agent.

Primary bacterial cultures of 5 from 19 nasal mucus samples were positive in RT-PCR on ASF, and their pool — in traditional ASF-PCR (Fig. 5). According to the microbiological analysis, the cultures from these samples contained *Pasteurella multocida*, *Neisseria pneumoniae*, and *Bacillus cereus* in association with *Candida albicans*. It should be noted that all 5 pigs had origin from one of the three homestead farms and were clinically sick with a mild form of PRDC: without fever and loss of appetite, but with 'morning cough' and weight loss; of these, two gilts died soon afterward without hemorrhages and other signs of ASF at autopsy. Subsequently, according to the regional State Service for Food Safety and Consumer Protection, the area with these householdings was recognized as an ASF outbreak according to the current procedure (<http://web.archive.org/web/20240622152134/https://pervom-rada.gov.ua/news/id/4613>).



**Figure 4.** Results of field monitoring of PRDC in one of the three farms where the associated form of ASF was detected: A — pig carcass with characteristic liver lesions for HA (necrotic spots), clear seropositivity for AD and weak positive reaction to ASF virus in the lateral test; B — pig carcass with characteristic lesions for PRDC (croupous pneumonia) with clear positive reaction to ASF virus in the lateral test; more details — in the text.



**Figure 5.** The result of detection of virophorus of the ASF pathogen (2a) in a pool of bacterial cultures of nasal mucus of pigs (1); more details — in the text.

**Discussion.** The basis of bacterial virophoria, at least concerning the AD pathogen, may be the adhesion of the corresponding bacterial receptors to the tegument of the outer envelope of the virion, which is formed from the cytoplasm of the cells of the biological host of the virus (Zimmerman et al., 2019). Another factor in the formation of bacterial virophoria may be physicochemical factors — magnetic irradiation (Brack et al., 1999), acidity (Buzun, Kolchyk, and Paliy, 2023), etc.

The first decade of the millennium saw important developments in the study of the associative activity of viruses. Researchers from the Institut Pasteur and CNRS demonstrated for the first time that certain viruses (like AID) can group into complex structures that resemble bacterial biofilms. These formations protect viruses from the immune system and greatly accelerate their spread between cells. It has been concluded that ‘viral biofilms’ are the main way of dissemination of viruses in the human body, in particular, acquired immunodeficiency viruses (AID), leukemia, and similar viruses (Pais-Correia et al., 2010; Gerilovych, Buzun and Kolchyk, 2013).

In the same period, Dr. Ribbeck from Harvard University revealed the role of bacterial virulence in the pathogenesis of viral diseases (Ribbeck, 2009; Pais-Correia et al., 2012). She proposed a mechanism by which viruses, essentially immobile particles and even more so *unable to move in a directed manner*, nevertheless exhibit tropism and are capable of spreading. According to her idea, in cells, the movement of viral particles is carried out by elements of the cytoskeleton, but *extracellular barriers*, such as mucus, etc., are overcome by viruses ‘hitchhiking on bacteria or sperm’.

Our data, together with the results of the Kharkiv Epizootiological School, add a general epidemiological dimension to the general pathogenetic significance of the associative activity of viruses and bacteria outlined above. After all, from the perspective of the ‘One Health’ paradigm, infectious diseases of humans and animals develop and spread by the same natural mechanisms, and the involvement of bacterial virophoria in enzootic processes in PRDC foci concerning AD and PCVI agents that we have identified cannot be interpreted separately as a phenomenon of PRDC alone, in isolation from general epidemiological patterns.

According to the literature, the highest association activity of PRDC viruses is observed with ‘respiratory’ bacteria of the genera *Pasteurella*, *Streptococcus*, *Glaesserella* (*Haemophilus*), *Actinobacillus*, *Bordetella*, as well as with mycoplasma of enzootic pneumonia (Opriessnig et al., 2011, 2012). However, we did not find any data on their virophoria against swine viruses. At the same time, the phenomenon of bacterial virophoria was studied at the NSC ‘IECVM’ on genetically related to AB virus — on herpesvirus of infectious rhinotracheitis and *Bacillus alvei* bacteria (Fuks, 1993, 1994, 1999). In addition, in the period 2015–2022, during the implementation of the relevant scientific topics, we often

observed the phenomenon of bacterial virophoria in the analysis of samples of pathological material, semen, and feed from pig premises contaminated with microbial associations containing circoviruses, parvoviruses, teschoviruses, viruses of Aujeszky’s disease, PRRS, endemic diarrhea and swine pox. These data became the basis for the formulation of the doctrine of switching the epizootic process of dangerous viral infections to the enzootic process and their rooting in pig production through the integration of their pathogen with the pig microbiota in the form of enzootic foci of reproductive and neonatal infections of pigs (Buzun et al., 2023). Therefore, the data obtained in the current study can be considered as a validation of this doctrine in a certain sense.

According to the obtained data (Fig. 3), the indices of the ratio of the odds of manifestation of virophoria (OR = 43.2) and the risks of exacerbation of PRDS (RR = 4.3) during the periods of activation and decrease of the incidence of PRDC in pigs, in our opinion, with the highest possible statistical probability, indicate that the associative activity of viruses and bacteria plays an important role in the formation and maintenance of the enzootic process of viral infections in pig production. At the same time, it may be a component of the processes of rooting exotic/emergent infections (research is ongoing).

Additional data on the virophoria of the ASF pathogen on bacteria from pig nasal mucus cultures in the PRDC enzootic focus are fully consistent with the data of Thai researchers and our experimental and clinical results on the possibility of integrating weakly virulent variants of asfarvirus with the consortium of PRDC pathogens within the ASF nosoarea (Havrylina and Evert, 2016; Assavacheep and Thanawongnuwech, 2022). Therefore, the enzootic process in ASF, as well as in reproductive and neonatal infections of pigs, including PRDC, is likely to be of the same nature as in AD and PCVI. That is, the formation of enzootic foci of ASF most likely occurs with the participation of associative interaction with bacteria of the pig’s microbiota in the form of virophoria.

Based on the above data, we propose a model for the rooting of exotic/emergent viral infections in pigs, in which certain species of bacteria in their microbiome and other factors suppress the pathogenicity of viruses and *transport them in the format of virophoria*, as interpreted by Ribbeck (2009), *to places of ‘hidden persistence’ of the virus* (in the body of wild boar, ticks, amoeba cysts, etc.) during the inter-epizootic period of the disease. During the exacerbation of an enzootic viral infection, certain factors will increase the pathogenicity of viruses, and bacteria (possibly of other species) in the same *virophoria format contributing to their massive channeling* into veterinary surveillance facilities, and thus to the activation of an enzootic outbreak in pig production.

Considering the importance of the problem of eradication of enzootic centers of animal viral infections, we consider it necessary to intensify the study of the role of bacterial virophoria as a driving force of the enzootic

process of emerging viruses and the search for innovative approaches to its use in anti-epizootic work — first of all against ASF. In this regard, it would be particularly valuable to study the presence of the phenomenon of bacterial virophoria and protists of domestic and wild pigs in the ASF world natural area — in Africa. In particular, the Republic of Nigeria has the necessary scientific and laboratory facilities, professional potential, and wide opportunities for fieldwork.

**Conclusion.** 1. On the model of two PRDS enzootic foci, by studying 183 samples of primary bacterial cultures from samples of clinical and pathological materials collected from pigs during periods of exacerbation and decrease in the activity of the PRDS enzootic process with the participation of PCV1 and AD pathogens, an active role of bacterial virophoria against these viruses in the activation of the enzootic process in PRDC foci in commercial pig production was established at a probability level of 99.99% ( $n = 240$ ). The indices of the ratio of the odds of an outbreak of this disease (OR) and the risk of its occurrence with the participation of

bacterial virophoria (RR) were  $16.12 \leq OR = 43.17 \leq 134.05$  and  $3.13 \leq RR = 4.25 \leq 5.77$ , respectively.

2. An algorithm has been developed for the evidence-based investigation of the existence of a mechanism of activation of the PRDS enzootic focus involving bacterial virophoria against the ASF pathogen. The data are not complete enough to conclude in this area.

3. Based on the obtained data, in the development of the Kharkiv direction of research on associated infections, a hypothesis of rooting of pathogens and maintenance of the enzootic process of exotic/emergent viral infections of pigs through the interaction of their etiological agents and causal factors with the manifestation of the phenomenon of bacterial virophoria was developed.

4. It is necessary to intensify the study of the role of bacterial virophoria as a component of the ASF enzootic process in order to develop innovative approaches to its use in anti-epizootic work: in particular, at the objects of veterinary and sanitary examination and the pig feed chain.

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