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Properties of *Actinobacillus pleuropneumoniae* isolates

V. A. Evgrafova, O. V. Pruntova, N. B. Shadrova, A. M. Timina
FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

SUMMARY

Results of tests of six *Actinobacillus pleuropneumoniae* isolates recovered from the diseased pigs kept in animal holdings located in the Russian Federation for their biological properties (biochemical, proteomic, antigenic and pathogenic ones) are presented in the paper. Proteomic properties were determined with mass-spectrometry using Autof MS 1000 mass-spectrometer (Autobio Diagnostics Co., Ltd, China): protein profiles were plotted and the peaks characteristic for each isolate were identified. Mass-spectra of tested *Actinobacillus* isolates and reference *Actinobacillus pleuropneumoniae* DSM 13472 strain were found to be in the m/z range of 2,000–12,000 Da. The following peaks (m/z) were common for all *Actinobacillus pleuropneumoniae* isolates and the strain: 2,541 ± 2; 4,267 ± 2; 5,085 ± 2; 6,450 ± 2; 7,207 ± 4; 9,408 ± 3; 11,820 ± 6. Therewith, the highest intensity (100%) was reported for the peak at 5,085 ± 2, that was supposed to be a specific feature of *Actinobacillus pleuropneumoniae*. All isolates were confirmed to belong to *Actinobacillus pleuropneumoniae* species and to 2, 5 and 9 serotypes by real-time polymerase chain reaction using species-specific and serotype-specific primers. *Actinobacillus pleuropneumoniae* isolates were tested for their pathogenic properties by experimental infection of white mice and 2.5–3 month-old piglets. All tested isolates were pathogenic for both white mice and piglets. Isolate No. 4 belonging to serotype 5 was found to be the most virulent out of tested isolates. Thus, LD_{50} was 4.19 lg microbial cells for white mice and 5.49 lg microbial cells for piglets that was consistent to the data of other authors carried out tests of actinobaccilli isolated in the Russian Federation for their pathogenicity. The isolates were deposited to the FGBI "ARRIAH" Collection of Microorganism Strains.

Keywords: porcine (*Actinobacillus*) pleuropneumonia, isolate, properties, polymerase chain reaction, mass-spectrometry, pathogenicity

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For correspondence: Olga V. Pruntova, Doctor of Science (Biology), Professor, Chief Researcher, Information and Analysis Centre, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: pruntova@arriah.ru*.

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Свойства изолятов *Actinobacillus pleuropneumoniae*

В. А. Евграфова, О. В. Прунтова, Н. Б. Шадрова, А. М. Тимина
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

РЕЗЮМЕ

Представлены результаты изучения биологических свойств (биохимические, протеомические, антигенные и патогенные) 6 изолятов *Actinobacillus pleuropneumoniae*, выделенных от больных свиней в животноводческих хозяйствах Российской Федерации. Протеомические свойства определяли посредством масс-спектрометрического анализа с использованием масс-спектрометра Autof MS 1000 (Autobio Diagnostics Co., Ltd, Китай): были построены белковые профили и определены характерные пики для каждого изолята. Установлено, что все масс-спектры изучаемых изолятов актинобацилл и референтного штамма *Actinobacillus pleuropneumoniae* DSM 13472 находятся в диапазоне m/z 2000–12 000 Да. Для всех изолятов и штамма *Actinobacillus pleuropneumoniae* общими были пики m/z: 2541 ± 2; 4267 ± 2; 5085 ± 2; 6450 ± 2; 7207 ± 4; 9408 ± 3; 11 820 ± 6, при этом самая высокая интенсивность (100%) была зарегистрирована для пика 5085 ± 2, который, как предполагаем, можно считать исключительной особенностью *Actinobacillus pleuropneumoniae*. Принадлежность всех изолятов к виду *Actinobacillus pleuropneumoniae* и серотипам 2, 5 и 9 была подтверждена методом полимеразной цепной реакции в реальном времени с использованием видо- и серотип-специфичных праймеров. Патогенные свойства *Actinobacillus pleuropneumoniae* определяли при экспериментальном заражении белых мышей и поросят 2,5–3,0-месячного возраста. Все испытуемые изоляты были патогенны как для белых мышей, так и для свиней. Установлено, что из всех изучаемых изолятов наиболее высокая вирулентность характерна для изолята № 4, который относится к 5-му серотипу. Так, $ЛД_{50}$ для белых мышей составила 4,19 lg м. к., для поросят – 5,49 lg м. к., что согласуется с данными других авторов, проводивших исследования патогенности актинобацилл, выделенных на территории Российской Федерации. Изоляты депонированы в коллекцию штаммов микроорганизмов ФГБУ «ВНИИЗЖ».

Ключевые слова: актинобациллезная плевропневмония, изолят, свойства, полимеразная цепная реакция, масс-спектрометрия, патогенность

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Для корреспонденции: Прунтова Ольга Владиславовна, доктор биологических наук, профессор, главный научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: pruntova@arriah.ru.*

INTRODUCTION

Actinobacillus pleuropneumoniae (APP) causes porcine pleuropneumonia, a disease that is widespread in many countries of the world and in the Russian Federation. This highly contagious disease is characterized by sudden onset, short clinical course, fibrin-hemorrhagic lung lesions in acute form or cough and decreased weight gains in chronic form of the infection. Porcine pleuropneumonia often leads to the animal death. Pigs of all ages are susceptible to the disease but 2–4-month-old piglets are the most susceptible. Persistence is a distinctive feature of the disease associated with long-term carriage of the bacterium by animal [1, 2]. The severity of the disease depends on the several factors the most important of which are APP serotype, infective dose, concomitant infections and animal keeping conditions [3–5].

In recent decades, there has been a trend in the Russian Federation towards an increase in the number of porcine APP-infected holdings, which could be accounted for breeding animal import from Western Europe and Canada, and absence of porcine pleuropneumonia in the list of infections from which the live pigs imported into the Russian Federation shall be free according to veterinary requirements [6–8].

Currently, there are some vaccines for porcine pleuropneumonia prevention classified into bacterin-toxoid, toxoid and bacterin-based (whole-cell bacteria). Bacterins confer protection against homologous serovars but do not protect against infection with heterologous serovars [9–13]. Vaccines based on inactivated Apx toxins are effective for reducing the incidence and clinical manifestations associated with the infection, but they are not able to prevent colonization by the pathogen in the lungs and their use poses a potential threat of infection of the herd by asymptomatic carrier-animals [9, 11, 14]. The development of universal anti-porcine pleuropneumonia vaccine able to protect against all known serovars is a tough task due to the lack of cross-immunity. The best way to eradicate this disease is to isolate and identify the causative agent in a particular APP-infected holding, to test the agent for its biological properties, to manufacture a vaccine and to apply the said autogenic vaccine in the said holding. Autogenic vaccines for porcine pleuropneumonia control are used in such countries as France, USA, Canada, etc. [9, 15, 16]. Considering the above-said, testing of APP isolates recovered from diseased pigs kept in animal holdings of the Russian Federation for their biological properties is of great importance. The novelty of the study was as follows: to recover new APP isolates, to test them for their biological properties, to deposit them into the FGBI "ARRIAH" Collection of Microorganism Strains for further vaccine development.

The study was aimed at isolation of APP agents from diseased pigs in animal holdings of the Russian Federation, to test them for their biochemical, proteomic, antigenic and pathogenic properties.

MATERIALS AND METHODS

Bacteria isolates. The following APP isolates recovered from pigs with respiratory disorders kept in the pig holdings located in the Russian Federation were used:

No. 1 – *A. pleuropneumoniae* "AU-21" isolate of serotype 2 recovered in the Kursk Oblast;

No. 2 – *A. pleuropneumoniae* "N-21" isolate of serotype 2 recovered in the Kursk Oblast;

No. 3 – *A. pleuropneumoniae* "VT-22" isolate of serotype 2 recovered in the Ryazan Oblast;

No. 4 – *A. pleuropneumoniae* "KG-21" isolate of serotype 5 recovered in the Belgorod Oblast;

No. 5 – *A. pleuropneumoniae* "OE-22" isolate of serotype 9 recovered in the Kursk Oblast;

No. 6 – *A. pleuropneumoniae* "DI-22" isolate of serotype 9 recovered in the Kirov Oblast.

Database of MALDI Autoflex III mass-spectrometer (Bruker Daltonik GmbH, Germany). Mass-spectrum of reference *A. pleuropneumoniae* DSM 13472 strain.

Test animals. APP isolates were tested for their pathogenic properties in white mice weighing 16–18 g and in 2.5–3.0 month-old piglets delivered from infectious disease-free holdings.

All tests in animals were carried out in strict compliance with intergovernmental standards on laboratory animal keeping and handling adopted by the Intergovernmental Council for Standardization, Metrology and Certification as well as in accordance with Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

Nutrient media and reagents. Brain-heart infusion agar (Becton, Dickinson and Company, USA) containing 5% of equine serum (AO "NPO "Microgen", Russia), 10% of yeast extract (FGBI "ARRIAH", Russia) was used for isolation

Fig. 1. Proteomic properties (protein profiles) of A. pleuropneumoniae isolates

of APP from pathological material samples. Commercial API NH test kit (bioMerieux, France) was used for testing the isolates for their biochemical properties.

Methods. Samples were collected in accordance with "Methodical guidelines for collection of biological material samples from animals for bacteriological tests"[1]. Bacteria were examined for their morphology by microscopy of Gram-stained smears. Bacteria were cultivated on dense agar media at temperature of (37.0 ± 0.5) °C for 24–48 hours.

APP were identified and tested for their proteomic properties using Autof MS 1000 mass-spectrometer (Autobio Diagnostics Co., Ltd, China). Single colonies of day-old culture were directly applied with sterile plastic loop on metal target plate. Saturated CHCA (*α*-cyano-4-hydroxycinnamic acid) solution prepared with 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid was used as a matrix. The device was calibrated daily using Calibrator Autobio Diagnostics reagent (Autobio Diagnostics Co., Ltd, China). Mass-spectrometric analysis of APP isolates was carried out using linear laser mode [17]. The analysis parameters were optimized for the m/z (mass/charge) range of 2,000 to 20,000 Da, the spectrum obtained by summing 20 single spectra with Auto Acquirer V2.0.130 software was recorded. Analysis of obtained mass-spectra was performed with Autof Analyzer V2.0.14 software (Autobio Diagnostics Co., Ltd, China).

Polymerase chain reaction was performed in accordance with the "Methodical guidelines for *Actinobacillus pleuropneumoniae* detection with polymerase chain reaction"[2].

APP isolates were tested for their pathogenic properties in white mice in accordance with the "Methodical guidelines for testing of the *Actinobaccillus pleuropneumoniae* antigens included in inactivated vaccines for their immunogenicity"[3]. Fifty white mice weighing 16–18 g (10 mice per each dilution) were used for tests of each isolate for

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[2] Scherbakov A. V., Timina A. M., Yakovleva A. S., Kovalishin V. F. Methodical guidelines for *Actinobacillus pleuropneumoniae* detection with polymerase chain reaction. No. 38-05. Vladimir: FGI "ARRIAH"; 2005. 8 p.

[3] Biryuchenkov D. A., Rusaleyev V. S., Frolovtseva A. A., Potekhin A. V. Methodical guidelines for testing of the *Actinobaccillus pleuropneumoniae* antigens included in inactivated vaccines for their immunogenicity. No. 69–08. Vladimir: FGI "ARRIAH"; 2008. 17 p.

its pathogenicity. The mice were infected intraperitoneally with day-old APP culture at the following concentrations: 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4 microbial cells/cm^3 in a volume of 0.5 cm^3. The mice were observed for 5 days.

lg LD_{50} was calculated according to Karber's formula modified by Ashmarin:

$$\lg LD_{50} = \lg D_N - \lg \delta (\Sigma L_i - 0.5),$$

where $\lg D_N$ – logarithm of maximum infective dose;

$\lg \delta$ – logarithm of the agent culture dilution factor;

L_i – dead test animal number/infected test animal number ratio;

ΣL_i – sum of L_i values for all tested doses.

APP isolates were tested for their pathogenic properties in 2.5–3.0 month-old piglets. The piglets were infected intratracheally with bacterium suspension containing 1×10^8 microbial cells in a volume of 1.0 cm^3. The piglets were observed for 10 days.

Microsoft Excel application and standard statistical methods were used for *statistical processing* of the data.

RESULTS AND DISCUSSION

The first stage of the study was the isolation of porcine pleuropneumonia agent from pathological material samples collected from dead and emergency slaughtered animals with respiratory disorders in the APP-infected holdings located in the Belgorod, Kirov, Kursk and Ryazan Oblasts. Six APP isolates were prepared for further study based on the tests results. The list of the said isolated is given above in "Materials and methods" section.

All tested isolates were shown to ferment glucose, sucrose, maltose, fructose and to be active against alkaline phosphatase, urease and *β*-galactosidase. Comparison of the obtained data with reference strain characteristics given in Bergey's Manual of Determinative Bacteriology [18] showed that characteristics of the said isolates determined with all tests were consistent with that ones of the reference strains indicating that the isolates belonged to APP species.

During the next stage of the study proteomic properties of all APP isolates were determined, protein profiles were plotted (Fig. 1) and peak mass lists enabling identification of specific peaks for each isolate were generated (Table 1).

Analysis of obtained results showed that all mass-spectra of tested *Actinobacillus* isolates and reference APP DSM 13472 strain were in the m/z range of 2,000–12,000 Da. The following peaks were common for all isolates and reference APP strain: m/z 2,541 ± 2; 4,267 ± 2; 5,085 ± 2; 6,450 ± 2; 7,207 ± 4; 9,408 ± 3; 11,820 ± 6, therewith, the highest intensity (100%) was recorded for peak of 5,085 ± 2 that was suggested to be a unique feature of APP.

The isolates were confirmed to belong to APP species by testing with real-time polymerase chain reaction (qPCR) with species-specific primers.

Figure 2 shows qPCR DNA amplification curves of tested isolate samples confirming that all samples belong to APP species [19–21].

Then, *A. pleuropneumoniae* isolates were serotyped using serotype-specific primers (Fig. 3–5).

Figure 3 shows qPCR DNA amplification curves of APP isolates No. 1, 2 and 3 confirming that they belong to serotype 2. DNA amplification curves of other isolates coincide with negative control curve indicating that they do not belong to serotype 2.

Table1
Analysis of mass-spectra of *A. pleuropneumoniae* isolates

m/z	Intensity (%) of *A. pleuropneumoniae* isolate and reference DSM 13472 strain spectra						
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	DSM 13472
2,541 ± 2	45	35	41	29	33	18	38
4,267 ± 2	56	53	61	48	49	22	70
5,085 ± 2	**100**	**100**	**100**	**100**	**100**	**100**	**100**
6,450 ± 2	52	51	54	55	51	43	67
7,207 ± 4	22	30	39	66	24	33	24
9,408 ± 3	14	25	29	38	13	22	26
11,820 ± 6	2	8	11	25	6	8	5

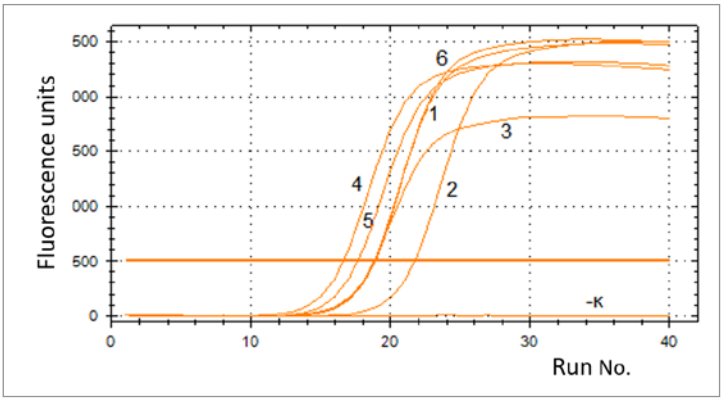

Fig. 2. Real-time PCR confirmation of isolate No. 1, 2, 3, 4, 5, 6 identification as A. pleuropneumoniae species ("-к" – negative control)

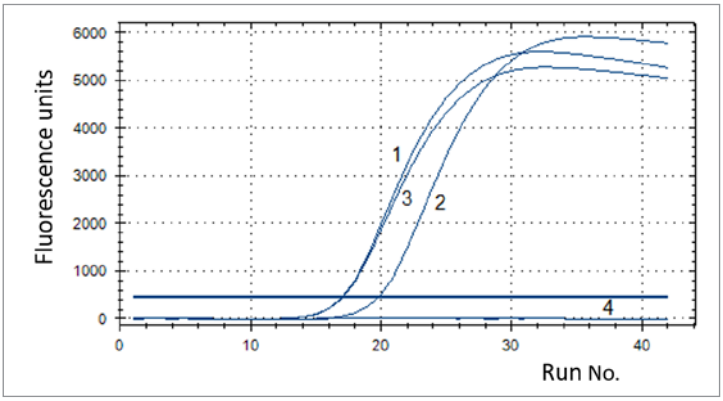

Fig. 3. Real-time PCR identification of A. pleuropneumoniae isolates as serotype 2 ones (1, 2, 3 – isolates No. 1, 2 and 3; 4 – isolate No. 4 and negative control)

Figure 4 shows qPCR DNA amplification curve of APP isolate No. 4 confirming that it belongs to serotype 5. DNA amplification curves of other tested isolates coincide with negative control curve indicating that they do not belong to serotype 5.

DNA amplification curves of serotype 9 APP isolate are shown in Figure 5. Isolates No. 5 and 6 were classified to this serotype based on qPCR results while amplification curves of other isolates coincide with negative control curve.

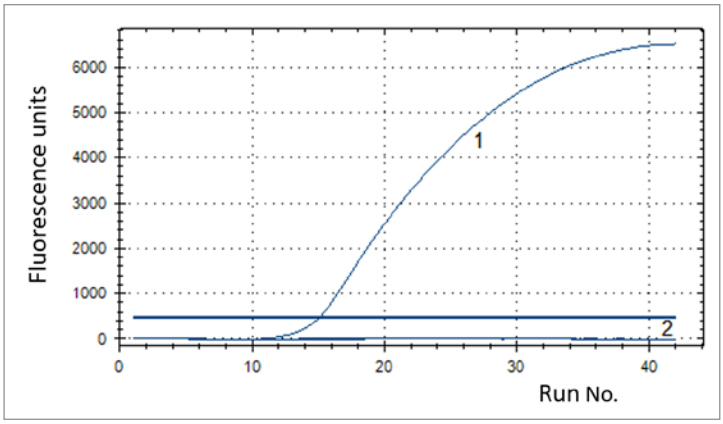

Fig. 4. Real-time PCR identification of A. pleuropneumoniae isolates as serotype 5 ones (1 – isolate No. 4; 2 – isolate No. 5 and negative control)

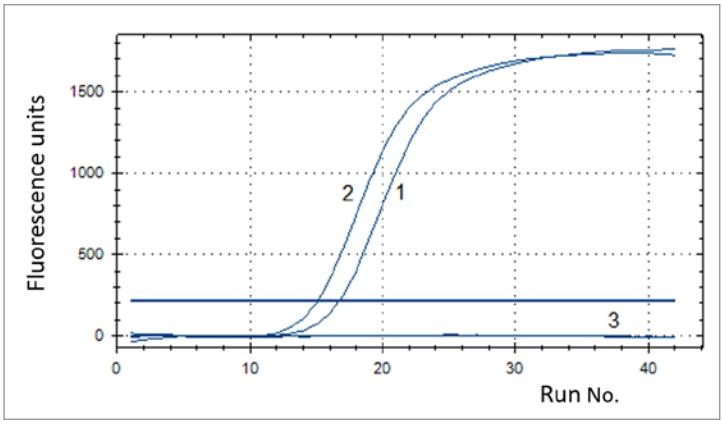

Fig. 5. Real-time PCR amplification of DNAs of serotype 9 A. pleuropneumoniae isolates (1 – isolate No. 5; 2 – isolate No. 6; 3 – isolate negative for serotype 9 and negative control)

Table 2
Pathogenic properties of serotype 2, 5 and 9 *A. pleuropneumoniae* isolates for laboratory and naturally susceptible animals

Animal species	lg LD_{50} microbial cells of isolates					
	serotype 2			serotype 5	serotype 9	
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
white mice	4.99	5.09	4.89	4.19	5.59	5.19
piglets	6.99	7.49	6.69	5.49	7.89	7.29

Thus, qPCR showed that tested APP isolates No. 1, 2 and 3 belonged to serotype 2, isolate No. 4 belonged to serotype 5 and isolates No. 5 and 6 belonged to serotype 9.

The next stage of the study was to test APP isolates for their pathogenic properties in laboratory and naturally susceptible animals. For this purpose, 50 laboratory mice weighing 16–18 g and 2.5–3.0 month-old piglets were experimentally infected in accordance with the above-mentioned method. All tested isolates were pathogenic for white mice and piglets since infected animals of all groups except for animals of control group died. The animal deaths were confirmed to be specific by recovery of pure cultures of tested isolates from brain-heart infusion agar with supplements (serum and yeast extract) inoculated with pathological materials collected from dead animals. White mice were observed for 5 days, piglets were observed for 10 days, observation results were recorded, interpreted. Table 2 shows lg LD_{50} values allowing comparative assessment of APP isolates for their virulence. Obtained data indicate that lg LD_{50} of the isolates for experimentally infected white mice was as follows: 4.99 ± 0.1 lg LD_{50} microbial cells for isolates No. 1, 2, 3 (serotype 2), 4.19 lg LD_{50} microbial cells for isolate No. 4 (serotype 5) and 5.39 ± 0.2 lg LD_{50} microbial cells for isolates No. 5 and 6 (serotype 9). lg LD_{50} of the isolates for experimentally infected 2.5–3.0 month-old piglets was found to be minimum (5.49 lg LD_{50} microbial cells) when isolate No. 4 (serotype 5) was used for inoculation and maximum (7.89 lg LD_{50} microbial cells) when isolate No. 5 (serotype 9) was used for inoculation that was indicative of high virulence of serotype 5 isolate.

Thus, isolate No. 4 belonging to serotype 5 was found to be higher virulent than other tested isolates, that was consistent with the data of other authors [1] carried out tests of actinobacilli isolated in the Russian Federation territory for their pathogenicity.

The isolates were deposited into the FGBI "ARRIAH" Collection of Microorganism Strains under the following names based on results of tests of these isolates for their biochemical, proteomic, serological and pathogenic properties:

– isolate No. 1 – *A. pleuropneumoniae* "AU-21" strain of serotype 2;
– isolate No. 2 – *A. pleuropneumoniae* "N-21" strain of serotype 2;
– isolate No. 3 – *A. pleuropneumoniae* "VT-22" strain of serotype 2;
– isolate No. 4 – *A. pleuropneumoniae* "KG-21" strain of serotype 5;
– isolate No. 5 – *A. pleuropneumoniae* "OE-22" strain of serotype 9;
– isolate No. 6 – *A. pleuropneumoniae* "DI-22" strain of serotype 9.

CONCLUSION

Tests of the bacterial cultures isolated in APP-infected pig holdings located in the Belgorod, Kirov, Kursk and Ryazan Oblast for their biological properties have showed that all tested isolates belong to *A. pleuropneumoniae* species. The following characteristic m/z peaks were determined based on tests of proteomic properties of the isolates: 2,541 ± 2; 4,267 ± 2; 5,085 ± 2; 6,450 ± 2; 7,207 ± 4; 9,408 ± 3; 11,820 ± 6 and were found to be similar to characteristics of reference *A. pleuropneumoniae* strain. The isolates were classified based on their antigenic properties as follows: isolates No. 1, 2 and 3 – to serotype 2; isolate No. 4 – to serotype 5; isolates No. 5 and 6 – to serotype 9. All isolates were pathogenic for laboratory and naturally susceptible animals. lg LD_{50} of the isolates for experimentally infected white mice was as follows: 4.99 ± 0.1 lg LD_{50} microbial cells of serotype 2 isolates, 4.19 lg LD_{50} microbial cells of serotype 5 isolate and 5.39 ± 0.2 lg LD_{50} microbial cells of serotype 9 isolates. The isolates were deposited to the FGBI "ARRIAH" Collection of Microorganism Strains based on the test results to be used for further anti-porcine pleuropneumonia vaccine development.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Valeria A. Evgrafova, Candidate of Science (Veterinary Medicine), Head of Laboratory for Bacterial Disease Prevention, FGBI "ARRIAH", Vladimir, Russia;
https://orcid.org/0000-0003-3053-6976, e-mail: evgrafova@arriah.ru.

Olga V. Pruntova, Doctor of Science (Biology), Professor, Chief Researcher, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0003-3143-7339, e-mail: pruntova@arriah.ru.*

Евграфова Валерия Андреевна, кандидат ветеринарных наук, заведующий лабораторией профилактики бактериальных болезней ФГБУ «ВНИИЗЖ», г. Владимир, Россия;
https://orcid.org/0000-0003-3053-6976, e-mail: evgrafova@arriah.ru.

Прунтова Ольга Владиславовна, доктор биологических наук, профессор, главный научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ» г. Владимир, Россия;
https://orcid.org/0000-0003-3143-7339, e-mail: pruntova@arriah.ru.

Natalya B. Shadrova, Candidate of Science (Biology), Head of Department for Microbiological Testing, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0001-7510-1269, e-mail: shadrova@arriah.ru.*

Anna M. Timina, Candidate of Science (Veterinary Medicine), Senior Researcher, Reference Laboratory for Highly Dangerous Diseases, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0002-0109-3507, e-mail: timina@arriah.ru.*

Шадрова Наталья Борисовна, кандидат биологических наук, заведующий отделом микробиологических исследований ФГБУ «ВНИИЗЖ», г. Владимир, Россия; *https://orcid.org/0000-0001-7510-1269, e-mail: shadrova@arriah.ru.*

Тимина Анна Михайловна, кандидат ветеринарных наук, старший научный сотрудник референтной лаборатории по особо опасным болезням ФГБУ «ВНИИЗЖ», г. Владимир, Россия; *https://orcid.org/0000-0002-0109-3507, e-mail: timina@arriah.ru.*