Scientific Works. Series C. Veterinary Medicine. Vol. LXX (2), 2024
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

Actinobacillus pleuropneumoniae PREVALENCE AND SEROTYPE DIVERSITY IN ROMANIAN PIG FARMS

Mădălina Iulia SITEAVU[1, 2], Roxana Ionela DRUGEA[1], Elena PITOIU[2], Emilia CIOBOTARU-PÎRVU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei Street, 050097, District 5, Bucharest, Romania
[2]Synevovet, 25 Industriilor Street, Chiajna, Ilfov County, Romania

Corresponding author email: madalinaiuliasiteavu@yahoo.com

Abstract

Actinobacillus pleuropneumoniae is a bacterial porcine respiratory tract pathogen that causes porcine pleuropneumonia, with high economic consequences and distribution all over the world. This study aimed to assess the prevalence of A. pleuropneumoniae in Romanian swine farms by two methods of diagnosis: microbiological examination and Real-time PCR. Serotyping was performed on 28 bacterial isolates from 6 farms. From 1281 number of tested samples by microbiological examination, there were obtained 137 number of isolates with a positive result for A. pleuropneumoniae, with an overall prevalence of 11%. By Real-time PCR, 231 samples were tested and 100 (43%) were positive for A. pleuropneumoniae, 13/81 (16%) lung tissue samples, and 87/150 (58%) oral fluid samples. The serotyping of 28 A. pleuropneumoniae-positive cultures revealed the presence of the following serotypes: 1-9-11, 2, 3, 4, 5, 14, the most frequently encountered being serotype 2, in 10 isolates (36%) and serotype 14, in 7 isolates (25%).

Key words*: A. pleuropneumoniae, swine, prevalence, serotyping.*

INTRODUCTION

Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*) is the causative agent of porcine pleuropneumonia, a disease that leads to severe economic losses in the swine industry all over the world, due to its high morbidity and mortality being one of the most important respiratory diseases in pigs (Vanni et al., 2012; Kucerova et al., 2011; Costa et al., 2011). *A. pleuropneumoniae* is considered a primary pathogen for Porcine Respiratory Disease Complex (PRDC), a multifactorial and polymicrobial disease that involves infectious factors, viral and bacterial, and non-infectious factors, such as genetics, environmental conditions, production system or management (Hansen et al., 2010; Dayao et al., 2014; van Dixhoorn et al., 2021).

The disease has different clinical forms, from peracute to subacute or chronic (Gómez-Laguna et al., 2014). *A. pleuropneumoniae* induces severe and rapidly fatal fibrinohemorrhagic and necrotizing pleuropneumonia, often detected in the postmortem inspection (Kamimura et al., 2016; Yoo et al., 2014). Animals that recover from acute infection and chronically infected animals can carry the pathogen in the nasal cavities and tonsillar crypts, becoming a source of infection and making eradication difficult (Hölzen et al., 2021).

A. pleuropneumoniae is classified as belonging to the family *Pasteurellaceae*, genus *Actinobacillus,* and is a Gram-negative, nonmotile, encapsulated, and facultative anaerobic bacteria (Pascu et al., 2022; Vanni et al., 2012). *A. pleuropneumoniae* isolates are classified based on the nicotinamide adenine dinucleotide (NAD) requirement for *in vitro* growth into biotype I (NAD-dependent) and biotype II (NAD-independent) (Zimmerman et al., 2019). There have been recognized 19 serotypes of *A. pleuropneumoniae*, based on differences in the antigenic properties of the capsular polysaccharides (Hernández-Cuellar et al., 2022; Stringer et al., 2021). Most serovars carry either the ApxI and ApxII toxin genes, which are considered more virulent, or the ApxII and ApxIII toxin genes, and in addition, all serovars carry the ApxIV toxin gene (MacInnes et al., 2008; Frey, 2019). The Apx toxins are serovar-dependent and have haemolytic and cytotoxic effects, leading to the

development of specific necrotic lung lesions (Stringer et al., 2021; Frey, 2019). ApxI is produced by serovars 1, 5a, 5b, 9, 10, 11, 14, and 16 and has strongly haemolytic and strongly cytotoxic effects; ApxII is present in all serovars except for 10 and 14, with weakly haemolytic cytotoxic effects; ApxIII is present in serovars 2, 3, 4, 6, 8, and 15, with strongly cytotoxic effects, but non-haemolytic effects; ApxIV is not characterized with haemolytic or cytotoxic effects (Sassu et al., 2018). The discovery of *A. pleuropneumoniae* toxins improved the diagnostic approaches and vaccine development for the disease (Frey, 2019).

The aim of this study was to assess the prevalence of *A. pleuropneumoniae* and its serotypes by different diagnosis methods originating from Romanian swine farms.

MATERIALS AND METHODS

Sampling. The study was conducted on samples collected between 2017 and 2022, and sent to the Synevovet laboratory. Swabs collected from affected lung tissue were used for microbiology testing. For molecular biology, lung tissues as well as oral fluid samples were analyzed. The farms are located all over the country.

The number of samples included in the study, per method, is presented in Table 1.

Table 1. Number of samples tested, per method

Microbiology	Molecular Biology	Coagglutination Serotyping
1281	**231 (81 lung tissues + 150 oral fluid)**	**28**

Isolation and Identification

Bacteriological examination. For sample culture, chocolate agar and blood agar mediums were used and incubated in anaerobic conditions (CO_2 5% thermostat) at 35-37 °C for 20-24 h. The colonies were selected based on their morphological characteristics and then identified using MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of flight mass spectrometry) technology.

Molecular biological examination. The samples were analyzed by Real-time PCR in laboratories from Spain, Germany, and from July 2020 in the Synevovet laboratory. In Synevovet laboratory, nucleic acid extraction was performed with the BioExtract Column Kit or the BioExtract Superball Kit (BioSellal, France). The viral identification was obtained using EXOone *Actinobacillus pleuropneumoniae* (Exopol, Spain), according to the manufacturer's instructions. For the amplification it was used the AriaMx Real-Time PCR System (Agilent, United States).

Serotyping. 28 positive cultures from 6 farms were selected for serotyping, which was performed in Spain by the coagglutination method and tested for serotypes 1–15.

RESULTS AND DISCUSSIONS

To assess the prevalence, two methods of diagnosis have been used: microbiological examination, a culture-dependent approach, and DNA identification by Real-time PCR. Bacteriological examination of affected lung tissue was the most frequently used method of diagnosis for this pathogen, and a number of 1281 samples were tested by this method. 137 isolates were positive for *A. pleuropneumoniae*, with an overall prevalence of 11%. The occurrence over the study period is presented in Table 2.

Table 2. Prevalence of *A. pleuropneumoniae* isolates over the study period

	2017	2018	2019	2020	2021	2022	Total
No. of tested samples	102	113	85	252	355	374	**1281**
No. of positive isolates	13	14	10	15	30	55	**137**
%	**13**	**12**	**12**	**6**	**8**	**15**	**11**

From 231 tested samples by Real-time RT-PCR, 100 (43%) were positive for *A. pleuropneumoniae*, 13 of 81 (16%) lung tissue samples, and 87 of 150 (58%) oral fluid samples (Figure 1).

The serotyping of 28 *A. pleuropneumoniae* isolates revealed the presence of the following serotypes: 1-9-11, 2, 3, 4, 5, and 14. The distribution of the serotypes is presented in Figure 2.

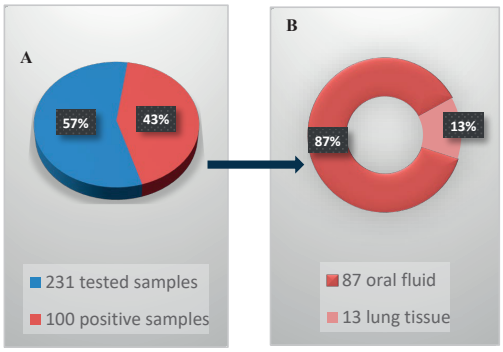

Figure 1. Total prevalence by Real-time PCR (A) and the distribution of positive samples by sample types (B)

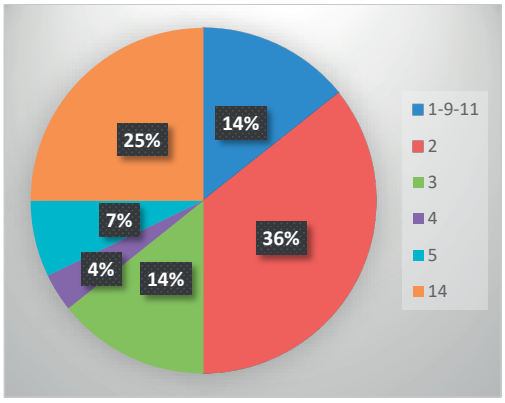

Figure 2. Serotypes revealed by typing the positive *A. pleuropneumoniae* isolates

Serotype 2 was detected in 10 isolates (36%), serotype 14 in 7 isolates (25%), serotypes 1-9-11 and 3 in 4 isolates each (14%), serotype 5 in 2 isolates (7%) and serotype 4 in 1 isolate (4%).

Serotype distribution among the farms is presented in Table 3. Four farms were positive for two or more serotypes, and two farms were positive only for serotype 2.

Table 3. Serotypes distribution among the farms

Farm	A	B	C	D	E	F
Serotype	3 and 14	2 and 1-9-11	2 and 4	2, 5, and 1-9-11	2	2

The overall prevalence was higher by Real-time PCR (43% vs. 11%). The occurrence of *A. pleuropneumoniae* in lung tissue samples was similar by microbiological examination (11%) and Real-time PCR (16%). The highest prevalence was shown in the last year of study.

The comparison of *A. pleuropneumoniae* detection by the same methods used in our study was performed in a study that tested tonsil samples, and positive results were obtained by PCR in all 12 tested pigs, but the isolation was possible in only nine samples by bacteriological examination (Chiers et al., 2002).

The high prevalence in oral fluid samples (58%) indicates a high number of carrier animals; this type of sample is also frequently used for monitoring purposes. For living animals, nasal swabs or tonsillar scraping were considered for bacteriological examination, but *A. pleuropneumoniae* resides deep in the tonsillar crypts, and commensal bacteria tend to overgrow it (Sassu et al., 2018). Bacterial detection to confirm a carrier state is not a method of choice (Gottschalk, 2015).

The distribution of *A. pleuropneumoniae* serotypes is very diverse around the world, and researchers from different countries have revealed data on the presence and prevalence of the corresponding *A. pleuropneumoniae* serotypes. Several studies indicate serotype 2 as the most prevalent in Europe (Sárközi et al., 2018; Soto Perezchica et al., 2023). Similar to our results, in a study from Italy, conducted from 2015 to 2022, the serotypes 9/11 (39.2%) and 2 (28.1%) are the most prevalent, with an increase of up to nine different serotypes isolated in the final study period (Guarneri et al., 2024). Serovar 2 was also the most prevalent (64%) in a study from Germany, followed by serovar 9/11 with about 15% of the isolates, and serovars 5, 6, 7, 8, 12, and 13 together representing 12% of the isolates; serovars 16 and 18 were also reported (Schuwerk et al., 2021). In Hungary, from 91 isolates, serotype 2 (39.5%) and serotype 13 (15.4%) were the most frequent (Sárközi et al., 2018). In Spain, the serotyping of biovar 1 isolates revealed that the most prevalent was serovar 4 (42.1%), then serovars 2 (24.3%), 9 (9.1%), and 5 (8.8%), while after the serotyping of biovar 2, serovar 7 was the most frequently encountered (68.5%), followed by serovars 2 (4.7%), 4 (4.7%), and 11 (1.6%) (Maldonado et al., 2009). Serotypes 2 (41.0%) and 4 (40.2%) were the most prevalent in a different report from Spain (Gutierrezmartin et al., 2006).

In England and Wales, serovar 8 was the most prevalent (71.7%) in the 2008–2014 period, and serovars 2, 6, 7, and 12 were also present in a smaller amount, the distribution of serotypes being similar to the 1995-2007 period (Li et al., 2016).

In a study from Japan, 95% of serovars, in decreasing order, are 2, 1, and 5 (Koyama et al., 2007). In a study from Canada on 142 *A. pleuropneumoniae* isolates, 75% belonged to serotypes 7 and serotype 5; serotypes 12, 2, 1, 8, 15, 6, 13, and 15 were present, in decreasing order (Lacouture & Gottschalk, 2020). Serovar 1 (65.4%) was predominant in Taiwan, followed by serovars 2 (34.1%) and 5 (0.5%), while in Thailand serotypes 1, 9, or 11 were predominantly found (29%), followed by serotypes 3, 6, or 8 and serotype 5a (26% each) (Assavacheep et al., 2003).

The results support the hypothesis that the prevalence of *A. pleuropneumoniae* and its serotypes may vary within pig farms worldwide. Thus, it is important to establish the serotype distribution in the pig population so that the pathogen can be monitored by implementing immunoprophylaxis programs and adding newly recognized serotypes in the construction of new vaccines (Kim et al., 2016).

Therefore, biosecurity protocols, good management practices, implementation of immunoprophylactic vaccination, development of prophylactic strategies for medicines, and good antimicrobial treatment are the main measures to control the incidence of *A. pleuropneumoniae* in pig farms (Kuchiishi et al., 2023).

CONCLUSIONS

This study brings information about the *A. pleuropneumoniae* prevalence and serotype distribution across Romanian swine farms. Two different diagnosis techniques were used for the assessment of *A. pleuropneumoniae* infection: microbiological examination and molecular biological examination, with an overall higher prevalence by PCR (43% versus 11%). Serotypes 1-9-11, 2, 3, 4, 5, and 14 were found. Serotype 2 was encountered most frequently, followed by serotype 14. Regular serotype monitoring is advisable since it provides insights into the epidemiology of *A. pleuropneumoniae* and its pathogenic capacity, the virulence being different among the serotypes. The high prevalence of *A. pleuropneumoniae* strains identified in our study leads to the need for a much larger study for serotype assessment, that includes all the serotypes known up to date, to have a better understanding of the significance of this pathogen on pig health in our country, as well as the need to strengthen the knowledge for proper surveillance and control of this disease.

ACKNOWLEDGEMENTS

We would like to thank Synevovet Laboratory and its team for their contribution to this study.

REFERENCES

Assavacheep, P., Persson, M., Luengyosluechakul, S., Watanaphansak, S., Laohasinnarong, D., Pungkhun, P., & Wallgren, P. (2003). *Actinobacillus pleuropneumoniae* in Thai pig herds. Prevalence of serum antibodies and relation to performance. *Journal of Veterinary Medicine, Series B, 50*(8), 390–395.

Chiers, K., Donné, E., Van Overbeke, I., Ducatelle, R., & Haesebrouck, F. (2002). Evaluation of serology, bacteriological isolation and polymerase chain reaction for the detection of pigs carrying *Actinobacillus pleuropneumoniae* in the upper respiratory tract after experimental infection. *Veterinary Microbiology, 88*(4), 385–392.

Costa, G., Oliveira, S., Torrison, J., & Dee, S. (2011). Evaluation of *Actinobacillus pleuropneumoniae* diagnostic tests using samples derived from experimentally infected pigs. *Veterinary Microbiology*, *148*(2–4), 246–251.

Dayao, D. A. E., Gibson, J. S., Blackall, P. J., & Turni, C. (2014). Antimicrobial resistance in bacteria associated with porcine respiratory disease in Australia. *Veterinary Microbiology, 171*(1–2), 232–235.

Frey, J. (2019). Rtx toxins of animal pathogens and their role as antigens in vaccines and diagnostics. *Toxins*, *11*(12), 719.

Gómez-Laguna, J., Islas, A., Muñoz, D., Ruiz, Á., Villamil, A., Carrasco, L., & Quezada, M. (2014). Infection dynamics and acute phase response of an *Actinobacillus pleuropneumoniae* field isolate of moderate virulence in pigs. *Veterinary Microbiology, 173*(3–4), 332–339.

Gottschalk, M. (2015). The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. *The Veterinary Journal*, *206*(1), 30–38.

Guarneri, F., Romeo, C., Scali, F., Zoppi, S., Formenti, N., Maisano, A. M., Catania, S., Gottschalk, M., & Alborali, G. L. (2024). Serotype diversity and

antimicrobial susceptibility profiles of *Actinobacillus pleuropneumoniae* isolated in Italian pig farms from 2015 to 2022. *Veterinary Research, 55*(1), 48.

Gutierrezmartin, C., Blanco, N., Blanco, M., Navas, J., & Rodriguezferri, E. (2006). Changes in antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Spain during the last decade. *Veterinary Microbiology, 115*(1–3), 218–222.

Hansen, M. S., Pors, S. E., Jensen, H. E., Bille-Hansen, V., Bisgaard, M., Flachs, E. M., & Nielsen, O. L. (2010). An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. *Journal of Comparative Pathology, 143*(2–3), 120–131.

Hernández-Cuellar, E., Guerrero-Barrera, A. L., Avelar-González, F. J., Díaz, J. M., Chávez-Reyes, J., & Salazar De Santiago, A. (2022). Adhesion mechanisms of *Actinobacillus pleuropneumoniae* to the porcine respiratory system and biofilm formation. *Austral Journal of Veterinary Sciences, 54*(3), 93–102.

Hölzen, P., Warnck, T., Hoy, S., Schlegel, K., Hennig-Pauka, I., & Gaumann, H. (2021). Comparison of protectivity and safety of two vaccines against *Actinobacillus pleuropneumoniae* in a field study. *Agriculture, 11*(11), 1143.

Kamimura, S., Sameshima, T., & Ito, H. (2016). Serovar and antimicrobial resistance profiles of *Actinobacillus pleuropneumoniae* isolated in Japan from 2006 to 2011. *Japan Agricultural Research Quarterly, 50*(1), 73–77.

Kim, B., Hur, J., Lee, J. Y., Choi, Y., & Lee, J. H. (2016). Molecular serotyping and antimicrobial resistance profiles of *Actinobacillus pleuropneumoniae* isolated from pigs in South Korea. *Veterinary Quarterly, 36*(3), 137–144.

Koyama, T., To, H., & Nagai, S. (2007). Isolation of *Actinobacillus pleuropneumoniae* serovar 15-like strain from a field case of porcine pleuropneumonia in Japan. *Journal of Veterinary Medical Science, 69*(9), 961–964.

Kucerova, Z., Hradecka, H., Nechvatalova, K., & Nedbalcova, K. (2011). Antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolates from clinical outbreaks of porcine respiratory diseases. *Veterinary Microbiology, 150*(1–2), 203–206.

Kuchiishi, S. S., Ramos Prigol, S., Bresolin, E., Fernandes Lenhard, B., Pissetti, C., García-Iglesias, M.-J., Gutiérrez-Martín, C.-B., Martínez-Martínez, S., Kreutz, L. C., & Frandoloso, R. (2023). Brazilian clinical strains of *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*: Capsular diversity, antimicrobial susceptibility (In vitro) and proof of concept for prevention of natural colonization by multi-doses protocol of tildipirosin. *Antibiotics, 12*(12), 1658.

Lacouture, S., & Gottschalk, M. (2020). Distribution of *Actinobacillus pleuropneumoniae* (From 2015 to June 2020) and *Glaesserella parasuis* (From 2017 to June 2020) serotypes isolated from diseased pigs in Quebec. *The Canadian Veterinary Journal = La Revue Veterinaire Canadienne, 61*(12), 1261–1263.

Li, Y., Bossé, J. T., Williamson, S. M., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N., & Langford, P. R. (2016). *Actinobacillus pleuropneumoniae* serovar 8 predominates in England and Wales. *Veterinary Record, 179*(11), 276–276.

MacInnes, J. I., Gottschalk, M., Lone, A. G., Metcalf, D. S., Ojha, S., Rosendal, T., Watson, S. B., & Friendship, R. M. (2008). Prevalence of *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Haemophilus parasuis*, *Pasteurella multocida*, and *Streptococcus suis* in representative Ontario swine herds. *Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire, 72*(3), 242–248.

Maldonado, J., Valls, L., Martínez, E., & Riera, P. (2009). Isolation rates, serovars, and toxin genotypes of nicotinamide adenine dinucleotide-independent *Actinobacillus pleuropneumoniae* among pigs suffering from pleuropneumonia in Spain. *Journal of Veterinary Diagnostic Investigation, 21*(6), 854–857.

Pascu, C., Costinar, L., & Herman, V. (2022). Antibiotic resistance of *Actinobacillus pleuropneumoniae* strains in the period 2016-2022 in Romania. *Romanian Review of Veterinary Medicine, 32*(4), 40–46.

Sárközi, R., Makrai, L., & Fodor, L. (2018). *Actinobacillus pleuropneumoniae* serotypes in Hungary. *Acta Veterinaria Hungarica, 66*(3), 343–349.

Sassu, E. L., Bossé, J. T., Tobias, T. J., Gottschalk, M., Langford, P. R., & Hennig-Pauka, I. (2018). Update on *Actinobacillus pleuropneumoniae* -knowledge, gaps and challenges. *Transboundary and Emerging Diseases, 65*, 72–90.

Schuwerk, L., Hoeltig, D., Waldmann, K.-H., Valentin-Weigand, P., & Rohde, J. (2021). Sero- and apx-typing of German *Actinobacillus pleuropneumoniae* field isolates from 2010 to 2019 reveals a predominance of serovar 2 with regular apx-profile. *Veterinary Research, 52*(1), 10.

Soto Perezchica, M. M., Guerrero Barrera, A. L., Avelar Gonzalez, F. J., Quezada Tristan, T., & Macias Marin, O. (2023). *Actinobacillus pleuropneumoniae*, surface proteins and virulence: A review. *Frontiers in Veterinary Science, 10*, 1276712.

Stringer, O. W., Bossé, J. T., Lacouture, S., Gottschalk, M., Fodor, L., Angen, Ø., Velazquez, E., Penny, P., Lei, L., Langford, P. R., & Li, Y. (2021). Proposal of *Actinobacillus pleuropneumoniae* serovar 19, and reformulation of previous multiplex PCRs for capsule-specific typing of all known serovars. *Veterinary Microbiology, 255*, 109021.

van Dixhoorn, I. D. E., Te Beest, D. E., Bolhuis, J. E., Parmentier, H. K., Kemp, B., van Mourik, S., Stockhofe-Zurwieden, N., van Reenen, C. G., & Rebel, J. M. J. (2021). Animal-based factors prior to infection predict histological disease outcome in Porcine Reproductive and Respiratory Syndrome Virus- and *Actinobacillus pleuropneumoniae*-infected pigs. *Frontiers in Veterinary Science, 8*, 742877.

Vanni, M., Merenda, M., Barigazzi, G., Garbarino, C., Luppi, A., Tognetti, R., & Intorre, L. (2012). Antimicrobial resistance of *Actinobacillus pleuropneumoniae* isolated from swine. *Veterinary Microbiology, 156*(1–2), 172–177.

Yoo, A. N., Cha, S. B., Shin, M. K., Won, H. K., Kim, E. H., Choi, H. W., & Yoo, H. S. (2014). Serotypes and antimicrobial resistance patterns of the recent Korean *Actinobacillus pleuropneumoniae* isolates. *Veterinary Record, 174*(9), 223–223.

Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J., Stevenson, G. W., & Zhang, J. (Ed.). (2019). *Diseases of swine* (*Eleventh edition*). Wiley-Blackwell/American Association of Swine Veterinarians.

CLINICAL SCIENCES