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POLYMICROBIAL AETIOLOGY OF SEVERE RESPIRATORY DISTRESS IN SWINE: A CASE STUDY FROM WESTERN ROMANIA

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

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, 5th District, 050097, Bucharest, Romania
[2]Synevovet Laboratory, 25 Industriilor Street, Chiajna, 077040, Ilfov County, Romania

*Corresponding author email: madalinaiuliasiteavu@yahoo.com

Abstract

The porcine respiratory disease complex represents a common polymicrobial condition that significantly impacts the worldwide swine industry. This study focused on identifying respiratory pathogens on a swine farm in Western Romania, where animals exhibited severe respiratory distress. A post-mortem examination was conducted on-site, followed by tissue sample and swab collection. Actinobacillus pleuropneumoniae, Pasteurella multocida, and Streptococcus suis were isolated. Real-time PCR analysis was conducted on five pooled samples, detecting Actinobacillus pleuropneumoniae in all of them. Alphainfluenzavirus influenzae (Swine influenza virus) was identified in two pools, while Circovirus porcine3 (Porcine circovirus 3) was present in one. All tested samples were negative for Betaarterivirus europensis (Porcine reproductive and respiratory syndrome virus), Mycoplasma hyopneumoniae, and Circovirus porcine2 (Porcine circovirus 2). Histopathology revealed necrotising haemorrhagic pneumonia or fibrinous suppurative bronchopneumonia. This study provides evidence of multiple pathogens in swine exhibiting severe respiratory distress, with Actinobacillus pleuropneumoniae as a predominant pathogen. The findings highlight the complex nature of porcine respiratory disease and underscore the need for targeted interventions to enhance swine health and production.

Key words*: swine, respiratory disease, Actinobacillus pleuropneumoniae.*

INTRODUCTION

Respiratory disease is one of the swine industry's biggest problems because it is linked to major production losses. The aetiology is typically polymicrobial and caused by the interplay of bacteria and viruses (Fablet et al., 2012; Opriessnig et al., 2011). The pathogens causing the porcine respiratory disease complex (PRDC) can create serious health issues when triggered by environmental factors or management deficiencies, such as overcrowding, mixing different sources of animals, temperature, continuous flow, ventilation, and sanitation (Assavacheep & Thanawongnuwech, 2022; Brogden & Guthmiller, 2002).

PRDC is usually characterised by anorexia, growth retardation, dyspnoea, cough, and fever (Thacker, 2001). The rate of morbidity may range from 30% to 70%, and mortality is considered between 4% and 6% (Opriessnig et al., 2011). Lesions are often encountered in the cranioventral area of the lung, where it fails to collapse; discoloration and consolidation may be found. Histopathological findings usually include bronchopneumonia along with interstitial pneumonia (Hansen et al., 2010). Depending on how the pathogens involved in coinfections or superinfections interact, the dynamic polymicrobial infections that are found in PRDC may result in varying clinical outcomes (Assavacheep & Thanawongnuwech, 2022). Implementing diagnostic and preventive strategies in impacted farms is challenging due to PRDC's complexity (Eddicks et al., 2021). It is often unclear who is the main pathogen and which one acts as a facilitating factor for secondary agents or further infections (Haimi-Hakala et al., 2017).

Both primary and opportunistic invasions can be caused by bacterial pathogens. Bacteria like *Bordetella bronchiseptica, Mycoplasma hyopneumoniae*, and *Actinobacillus pleuropneumoniae* are considered primary bacterial agents. The most common opportunistic agent is *Pasteurella multocida*, and the infection with other common

opportunistic agents like *Glaesserella parasuis*, *Trueperella pyogenes*, *Streptococcus suis, Actinobacillus suis*, and *Salmonella choleraesuis* may potentially result in respiratory conditions (Brogden & Guthmiller, 2002; Saade et al., 2020). The most common viral agents involved in the PRDC are *Betaarterivirus europensis* (Porcine reproductive and respiratory syndrome virus, PRRSV), *Alphainfluenzavirus influenzae* (Swine influenza virus, SIV), *Varicellovirus suidalpha1* (Aujeszky's disease virus, ADV), and *Circovirus porcine2* (Porcine circovirus 2, PCV2) (Assavacheep & Thanawongnuwech, 2022; Zimmerman et al., 2019). The range of infectious agents can intensify and prolong symptoms by interacting with one another in a complicated and perhaps synergistic way (Saade et al., 2020).

The purpose of this study was the investigation of an acute outbreak of respiratory disease and the identification of the pathogens causing it, in a Romanian swine farm.

MATERIALS AND METHODS

Sampling

A large-scale growing pig farm with an intensive system from Western Romania was the site of the study. The disease had a sudden onset, with animals showing signs of severe respiratory disease, dyspnoea, cough, severe apathy, anorexia, high fever, and sudden death as well as nervous signs. On-site post-mortem examination was performed, and representative tissue samples (lymph nodes, lung, and brain fragments) and swabs were collected and sent to the laboratory. Laboratory diagnosis was performed in Synevovet, Romania.

Molecular biological examination

Lung tissues and tracheobronchial lymph nodes were tested in five pools, each pool corresponding to a shed, for PRRSV, PCV2, PCV3, *A. pleuropneumoniae*, *M. hyopneumoniae*, and SIV by real-time PCR. Nucleic acid extraction was performed with the BioExtract Column kit (BioSellal, France). The following qPCR kits were used for pathogen detection: Bio-T kit PRRSV, with the differentiation of European and North American genotypes; Bio-T kit PCV2& PCV3 (BioSellal, France); EXOone *Actinobacillus pleuropneumoniae*; EXOone *Mycoplasma hyopneumoniae*; and EXOone Influenza A virus (Exopol, Spain), in compliance with the manufacturer's guidelines. The nucleic acids were amplified using the AriaMx instrument for real-time PCR (Agilent, United States).

Bacteriological examination

25 swabs were collected from affected tissues: lung, pericardial sac, lymph nodes, and brain, and they were cultivated on blood agar and chocolate agar growth mediums. The incubation was done in anaerobic conditions (thermostat with 5% CO_2) at 35-37°C for 20- 24 h. Then the colonies were selected based on their morphological characteristics, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology was used to identify them.

Histopathology

During necropsy, lung samples were collected and then underwent a standard procedure involving 10% formalin fixation, paraffin embedding, sectioning, and H&E staining. Imaging and histological examination were performed using an Olympus CX23 microscope with an SC50 camera (Olympus, Tokyo, Japan).

RESULTS AND DISCUSSIONS

Reported gross findings include fibrinosuppurative, haemorrhagic, and necrotising pneumonia; interlobular oedema; and pleural adhesions (Figure 1, panels A to D). Laboratory diagnosis revealed the presence of several pathogens, viruses, and bacteria. By molecular biological examination, all the tested samples were positive for *A. pleuropneumoniae*, SIV was detected in two pools, and PCV3 was found in one pool. PRRSV-1, PRRSV-2, PCV2, and *M. hyopneumoniae* were not detected. From 16 isolates, five types of bacterial agents were identified by microbiological examination. *A. pleuropneumoniae*, *Escherichia coli* (*E. coli*), *Pasteurella multocida* (*P. multocida*), and *Streptococcus suis* (*S. suis*) were isolated from lung tissues; *Staphylococcus aureus* (*S. aureus*) and *E. coli* were present in the pericardium sac, and *E. coli* was also isolated from the brain tissue.

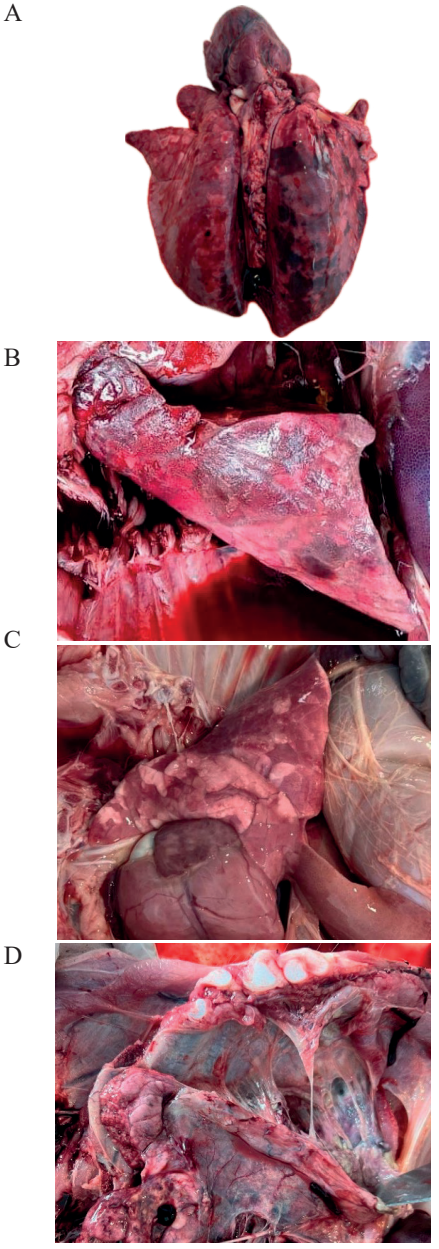

Figure 1. Gross pathological findings: (A) Deep red coagulative necrosis areas, sharply demarcated of fibrino-haemorrhagic and necrotising pneumonia; (B) Fibrinous exudate covering the pleural surface; (C) Cranioventral areas of consolidation; (D) Pleural adhesions

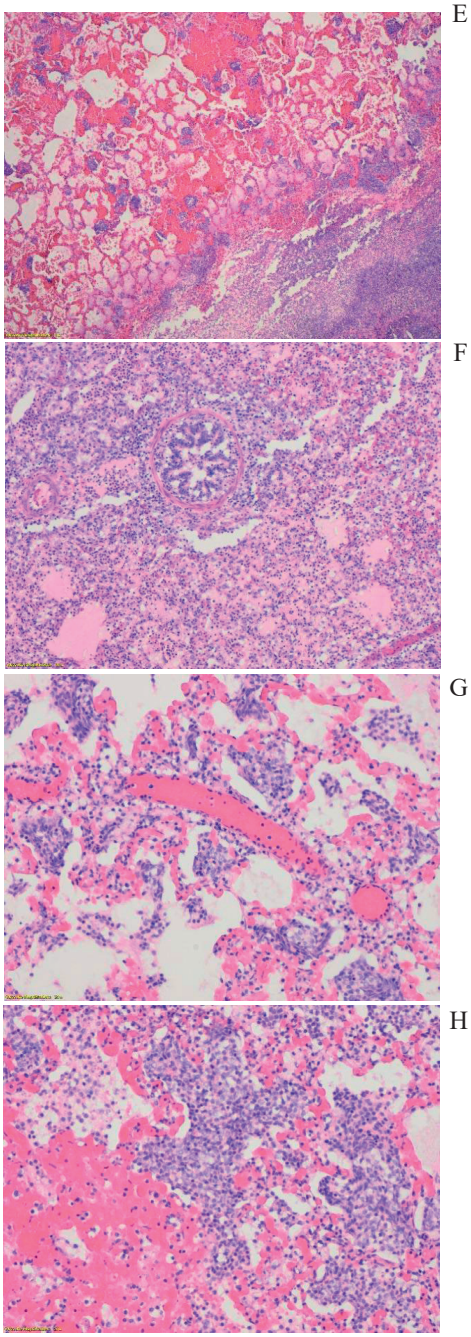

Figure 2. Microscopic appearance of lesions: (E) Lung, fibrinosuppurative bronchopneumonia and necrotising haemorrhagic pneumonia, H&E stain, 40x; (F) Lung, bronchiole infiltrated by inflammatory cells and filled with fibrinosuppurative exudate, H&E stain, 100x; (G) Lung, intra-luminal thrombosis of vessels (vasculitis), H&E stain, 200x; (H) Many degenerate neutrophils and macrophages admixed with abundant fibrin and necrotic cellular debris are filling and replacing alveoli and expanding interlobular septae. Alveoli contain oat cells, H&E stain, 200x

Histopathology revealed necrotizing haemorrhagic pneumonia fibrinous suppurative bronchopneumonia, with oat cell formation, fibrinonecrotizing vasculitis, and pleuritis (Figure 2, panels E to H). Table 1 summarises the pathogens detected in each shed, corresponding to qPCR detection and bacteriological examination.

Table 1. qPCR and bacteriological examination results corresponding to each shed

qPCR	*1*	*2*	*. 3*	*4*	*5*
PRRSV-1	Negative	Negative	Negative	Negative	Negative
PRRSV-2	Negative	Negative	Negative	Negative	Negative
PCV2	Negative	Negative	Negative	Negative	Negative
PCV3	Negative	Negative	Negative	**Positive (ct: 29)**	Negative
APP*	**Positive (ct: 31)**	**Positive (ct: 34)**	**Positive (ct: 24)**	**Positive (ct: 18)**	**Positive (ct: 29)**
*M.HYO**	Negative	Negative	Negative	Negative	Negative
SIV*	**Positive (ct: 39)**	Negative	Negative	**Positive (ct: 37)**	Negative
Bacteriological examination	*1*	*2*	*. 3*	*4*	*5*
APP*	Negative	Negative	**Positive**	**Positive**	**Positive**
P. multocida	Negative	**Positive**	**Positive**	**Positive**	Negative
S. suis	**Positive**	**Positive**	**Positive**	Negative	Negative
E. coli	**Positive**	**Positive**	Negative	Negative	**Positive**
S. aureus	Negative	Negative	Negative	**Positive**	Negative

**APP - Actinobacillus pleuropneumoniae; M.HYO - Mycoplasma hyopneumoniae; SIV - Alphainfluenzavirus influenzae (Swine Influenza Virus)*

The purpose of this investigation was to identify the pathogens causing an ongoing respiratory outbreak. One of the most important health issues that growing pigs face is respiratory infection (Hansen et al., 2010). Although most swine respiratory disease cases end in death due to bacterial pneumonia, the PRDC has a multifactorial causation, meaning that other infections and the interplay of pathogens, host background, and environmental variables play a significant role in its development. For effective control of the disease, all factors should be identified, including the underlying infectious cause, not only the actual cause of the deaths (Grant Maxie, 2016).

In the present study, many pathogens were present in the affected farm: two viruses, SIV and PCV3, and five bacteria, *A. pleuropneumoniae*, *S. suis*, *P. multocida*, *E. coli,* and *S. aureus*. In PRDC cases, four or more infectious agents are frequently found, single-pathogen positive rates being lower than those of multiple-pathogen combinations, leading to intricate and perhaps synergistic interactions that might worsen respiratory illness and lesions' severity and duration (Cheong et al., 2017; Zimmerman et al., 2019). In a retrospective study of the etiologic agents linked to respiratory illnesses in pigs in the United States, 88.2% of the cases examined included two or more infectious agents (Choi et al., 2003).

A. pleuropneumoniae was identified in all the sheds by qPCR, with cycle threshold (Ct) values between 18 and 34. In the shed with the highest *A. pleuropneumoniae* load, the bacteria were associated with SIV and PCV3; *S. aureus* was isolated from the same shed. It is considered that *A. pleuropneumoniae* can be a primary as well as a secondary pathogen (Brauer et al., 2012). Numerous factors can influence the *A. pleuropneumoniae* outbreaks, as well as the outcome of infection. The disease is greatly impacted by strain virulence and the presence of additional pathogens. For both acute and chronic (pleuritic) infections, a specific effect of the coinfection has been proposed (Fablet et al., 2012; Zimmerman et al., 2019).

In studies of coinfection between SIV and *A. pleuropneumoniae*, a considerably higher local cytokine response was reported (Czyżewska-Dors et al., 2017), and it has been demonstrated that the virus shedding and replication were higher in the coinfected pigs, with more severe symptomatology and lesions than the groups infected with one pathogen (Pomorska-Mól et al., 2017). An *in vitro* study of the coinfection between *A. pleuropneumoniae* and *S. suis* reported synergistic interactions between both pathogens and increased antibiotic resistance. When it was cultured along with *S. suis*, *A. pleuropneumoniae* developed without nicotinamide adenine dinucleotide and formed strong biofilms (Wang et al., 2020).

SIV was detected in two pools, with high Ct values (37 and 39). The virus is cleared in the lung tissue as fast as 72 hours postinfection (Grant Maxie, 2016), which may explain its inconsistent rates of detection. SIV is a key contributor to the PRDC, with an endemic course or rapidly spreading outbreaks of severe nonfatal disease (Grant Maxie, 2016). Other bacteria isolated in SIV-positive sheds included *S. suis*, *P. multocida*, *E. coli,* and *S. aureus*. Secondary bacterial infection is predisposed by the altered host response following SIV

infection due to complex mechanisms that would greatly raise the mortality rate (Lin et al., 2015). Coinfections between *S. suis* and the influenza virus are frequently encountered in clinical outbreaks (Lin et al., 2015). SIV infection can help *S. suis* colonise epithelial cells, being used as a vehicle, as bacteria adherence and cellular invasion were proven to improve when both infections evolve at the same time. SIV infection can also facilitate the entry of *S. suis* into the bloodstream through the respiratory tract. Additionally, during influenza infection, *S. suis* can enhance local inflammatory response in the respiratory system (Meng et al., 2015; Saade et al., 2020; Wang et al., 2013). In an *in vitro* study of the coinfection between SIV and *S. aureus*, the results showed a drastic enhancement in pathogeny, fatal disease, and extended lung lesions due to the virus haemagglutinin activation by the *S. aureus* protease (Tashiro et al., 1987). A diagnostic data study involving 2,872 pigs with respiratory disease identified SIV as the second most frequently detected pathogen, with 636 positive cases, of which SIV only was found in 89 (3.1%) samples, while the highest rate of coinfection was recorded with *P. multocida* in 148 (5.2%) (Choi et al., 2003).

PCV3 was identified in one pool, with a Ct value of 29. *A. pleuropneumoniae*, *P. multocida*, *S. suis,* and *S. aureus* were isolated from the same shed. PCV3 is present all over the world, in healthy and diseased herds (Opriessnig et al., 2020). A notable increase in PCV3 titres was observed in pigs with clinical disease development, compared to those without symptoms (Kedkovid et al., 2018). The connection between PCV3 infection and the occurrence of respiratory illness and lung damage has been documented in several studies (Kedkovid et al., 2018; Palinski et al., 2017; Phan et al., 2016; Savic et al., 2020; Shen et al., 2018; Zhai et al., 2017). Research, including those by Savic et al. (2020), Kedkovid et al. (2018), and Phan et al. (2016), has indicated that PCV3 co-infections with *A. pleuropneumoniae*, *P. multocida*, and *S. suis* are observed. PCV3 contributes to the disease development and the amplification of symptoms (Savic et al., 2020). Circoviruses interfere with the production of interferon and pro-inflammatory cytokines, thus affecting the immune response. Processes such as apoptosis, alteration of cell transport, and mitotic phase arrest also contribute to viral replication. Cytokine imbalance and lymphocyte depletion lead to weakened immunity, favouring the invasion of secondary or concurrent infections. This combination intensifies the severity of diseases associated with circoviruses (Fehér et al., 2023).

Pneumonia and septicaemia in pigs can be initiated by highly pathogenic *P. multocida* strains. *P. multocida* is regarded as a major contributor to respiratory issues and is considered the most frequently recovered bacteria in pigs suffering from pneumonia (Piva et al., 2023). *S. suis* coinfections are prevalent and can raise mortality rates by causing severe pneumonia (Lin et al., 2015). In general, animals with multiple bacterial infections, either co-occurring or following a primary infection, exhibit worse clinical symptoms, increased lung lesions, and decreased performance than those with single bacterial infections, alongside alterations in immune system responses (Saade et al., 2020). It is believed that bacterial development is favoured by viral infection of the respiratory system (Tashiro et al., 1987).

CONCLUSIONS

This study confirms the presence of multiple respiratory pathogens in swine exhibiting severe respiratory distress, with *A. pleuropneumoniae* as a predominant pathogen, SIV and its interactions with opportunistic bacteria, and PCV3, which might contribute to the severity of respiratory problems. Further research on lung diseases, as well as the impact of the interactions between the pathogens linked to these conditions on the severity of disease development, would lead to a better implementation of effective control measures. The findings highlight the complex nature of porcine respiratory disease and underscore the need for targeted interventions to enhance swine health and production.

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