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DETECTION OF THE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IN A ROMANIAN PIG-FATTENING FARM

DETECȚIA VIRUSULUI SINDROMULUI RESPIRATOR ȘI DE REPRODUCȚIE PORCIN ÎNTR-O ÎNGRĂȘĂTORIE DE PORCI DIN ROMÂNIA

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ABSTRACT

PRRS is one of the most burdensome morbid entities facing a pig-fattening farms. Although reported in few countries since the 1980s, now days PRRS virus/ disease have now been declared worldwide.

The purpose of this paper is to assess the presence of the porcine respiratory and reproductive syndrome virus (PRRSV) in a pig fattening with respiratory disease history, of undetermined aetiology, by using molecular biology diagnostic methods and whole blood samples. The samples were collected from 14 pigs, randomly chosen, belonging to recently purchased piggy and growing pigs. Detection of PRRSV was done by a conventional PCR method. The RNA extraction was performed using a commercial kit (QIA amp cador Pathogen Mini Kit, Qiagen), as prescribed by the manufacturer. The reaction mixture required by classical PCR technique was the one provided by the commercial kit "OneStep RT-PCR" (Qiagen). In the herd under investigation, swine proved to be infected with PRRSV. The results obtained are complying with the respiratory disease manifested in the farm, where 64.29% samples were tested positive for PRRSV specific amplicon. In the pigs tested the frequency of the positive samples was found to be higher in the growing pigs (>60 days old).

Keywords: PRRS, molecular biology, PCR

REZUMAT

Sindromul respirator și de reproducție porcin (PR RS) este una dintre cele mai importante entități morbide ce se întâlnește în crescătoriile de suine. Raportată în doar câteva țări în anii 1980, în prezent sunt declarate focare de PRRS în întreaga lume.

Scopul acestei lucrări îl reprezintă evaluarea prezenței virusului PRRS într-o îngrășătorie de porci cu istoric de afecțiuni respiratorii, cu etiologie necunoscută, folosindu-se tehnici de biologie moleculară și probe de sânge integral. Probele de sânge s-au recoltat de la 14 porci din categoria de tineret recent introdus în exploatație și categoria grăsuni. Detecția virusului PRRS s-a realizat printr-o metodă PCR clasică.Extracția ARN s-a efectuat cu ajutorul unui kit comercial: (QIAamp *cador* Pathogen Mini Kit, Qiagen), conform recomandărilor producătorului. Mix-ul de reacție necesar tehnicii PCR varianta clasică s-a efectuat tot cu un kit disponibil comercial (OneStep RT-PCR, Qiagen).

În exploatația investigată, suinele de la care s-au recoltat probe biologice prezentau sindrom respirator posibil asociat infecției cu PRRSV. În ferma cercetată ponderea probelor pozitive a fost 64,29% . Ponderea rezultatelor pozitive în efectivul testat, la respectiva serie, s-a dovedit a fi mai mare la categoria de vârstă 60 zile.

Cuvinte cheie: PRRS, biologie moleculară, PCR

Porcine respiratory and reproductive syndrome (PRRS) is one of the most important morbid entities challenging the swine industry, characterized by two main disorders: impairment of reproductive function and respiratory distress, up to the pig age (7, 15). Its reproductive impairment affect both females (sows and gilts) and boars, to the last ones causing contamination and depreciation of the semen quality, altered sexual behaviour and general condition. Respiratory syndrome occurs mainly in young pigs, but it can be also manifest in growing and breeding categories (6, 11).

Since the '80s, PRRS outbreaks have been declared worldwide, with both epidemic and/or endemic pattern (8). Over the past twenty years, studies concerning PRRSV seroprevalence (1, 2, 3, 4) and characterisation of Romanian PRRSV isolates (12, 13, 14) have been conducted in several Romanian farms.

Also, since the early 2000s, there were monitored the pathology of reproduction and respiratory syndrome (5, 9, 10). Seroprevalence recorded in Romania was variable in different farms [IgG PRRSV - 12% positive (3)] and several programs of control have been recommended (1, 2, 4).

However, despite control measures applied over the last twenty years, evidence of PRRSV persistence in several Romanian farms are still recorded. This may

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be due to both the biosecurity issues applied and the way in which diagnostic methods are used to monitor PRRSV circulation in and between farms. PRRSV antibodies can be detected with IFA, ELISA, and VN (4, 15). Considering virus detection, there have been described numerous PCR (e.g., classical PCR, real-time PCR, loop-mediated isothermal amplification) and viral antigens detection (e.g., immunohistochemistry, immunochromatography, immunofluorescence) techniques (15). In Romanian pig farms are used mainly serological assays (ELISA-Ab tests) and reverse transcriptase-polymerase chain reactions (RT-PCR) (1, 2, 3, 4, 12, 13, 14). Sensitivity and specificity of ELISA tests commercially available (e.g., ELISA Herd Chek PRRS, IDEXX) are high and able to detect infection (specific PRRSV-Ab) at 9-14 days post infection (DPI) and the history of exposure up to 12 months. However, serological assays can differentiate between infection and vaccination antibodies only if acute and convalescent serum samples are used, and the definitive diagnosis of PRRS will be achieved after a long awaited period (2, 4, 15). Also, RT-PCR techniques proved high sensitivity and specificity, with serum and most tissues positive from 1 to 251 DPI in persistently infected swine (15). One of the main disadvantages of PCR is inability to differentiate between infectious and non-infectious virus, which requires correlation of the laboratory result with epidemiological, clinical and anatomopathological data.

The purpose of this paper is to assess the presence of the porcine respiratory and reproductive syndrome virus (PRRSV) in a pig fattening with respiratory disease history, of undetermined aetiology.

MATERIALS AND METHODS

Collection, packaging and transportation of samples

The blood samples were collected from pigs belonging to Brăila County. The farm is a rearing unit. The pigs are purchased from a nursery unit, at the age of 60-63 days with the initial weight of 20-25 kg. The farm grows the pig until about 180 days and send them to the slaughterhouse at a weight of 110-120 kg. The pigs are housed in different halls, according to their age. In the farm, the respiratory signs were noticed from the 60-days of age to the 130-days age.

Blood samples were collected from both age category, from pigs housed in seven different compartments. The sampling was carried in accordance with the Sanitary Veterinary Norm on the Methodology of Sampling, Primary Processing, Packaging and Transport of Samples for Laboratory Examinations in the Field of Animal Health from 19.03.2008 of the National Sanitary Veterinary and Food Safety Authority - ANSVSA, published in The Official Monitor of Romania, in effect since May 2, 2008, Chapter VIII - "Samples for Virological, Serological, Immunological and Haematological Examinations".

For the intended laboratory test, it collected integral blood on EDTA. After sampling, the collecting tubes were gently stirred and then refrigerated at 2°C - 4°C, during the transport and into the lab unit, until test, in order to preserve the morphological integrity of the blood cells. Samples were numerically as below (Table 1).

Table 1

Identification data of the individuals blood samples

Sample no.	Age (days)	Gender
1	63	M
2	63	M
3	60	M
4	60	M
5	63	F
6	60	F
7	60	M
8	60	M
9	60	M
10	60	F
11	110	F
12	120	M
13	120	F
14	130	F

RNA extraction

The RNA extraction technique takes place in four stages: 1./ cell lysis; 2./membrane lipids removal; 3./ protein removal; 4./ precipitation.

In the present study RNA extraction was performed using the commercial kit: „QIAamp cador Pathogen Mini Kit" (Qiagen). The extraction was performed according to the manufacturer's recommendations.

„QIAamp cador Pathogen Mini Kit" simplifies the isolation of viral RNA and viral DNA as well as bacterial DNA from a whole series of biological samples, including integral blood, serum, tampons and tissues. Using a fast spin-column procedure, contaminants and inhibitors are removed to isolate the nucleic acids ready to use for applications such as classic and real-time PCR.

PCR technique

The reaction mix required for the classical PCR technique was prepared using the "OneStep RT-PCR, Qiagen" detection kit. To ensure repeatability of the reaction, all samples were operated in duplicate. In order to certify the method, a positive control represented by vaccine virus and a negative one, represented by ultrapure water, were used. The DNA fragment chosen for PRRS detection is from ORF 7 and the primers used are described in Table 2 (14).

The reaction mix include the following reagents: primer mix 50 pmol/µl; ultrapure water 14,8 µl; One Step RT-PCR buffer 5X 5,1µl; dNTP 10Mm; RNasin 40U/µl; enzyme mix OneStep RT-PCR (Qiagen)1,1 µl and 3 µl RNA for each sample. The PCR amplification protocol was: 48°C - 30 minutes 1 cycle; 95 C - 10 minutes 1 cycle; 95°C - 15 minutes, 60°C - 2 minutes, 40 cycles; 72°C – 7 minutes 1 cycle. Electrophoresis was performed in agarose gel (1.5% agarose in TAE) - 0.9 g agarose + 60 ml TAE 1x + 5 µl EtBr 10 mg / ml. Migration: 100V; 1,5A; 30 min. 12 µl EtBr 10 mg/ml is added to the migration buffer. The size of the sought DNA is 291 bp.

Table 2

Primer sequences for PRRS detection

Primer PRRS - 2	5 µM	5'- GCGAATCAGGCGCACWGTATG - 3'
Primer PRRS - 4	5 µM	5'- AGAAAAGTACAGCTCCGATGG - 3'

RESULTS

The electrophoretic image generated by the UV reader, revealed the presence of the specific amplicon for the PRRSV genome at 64.29% of the samples, white lanes of different intensity in Figure 1 corresponding to the sought amplicon, respectively proving the presence of the PRRSV genome in the investigated sample.

Using the classical PCR method for the present study, the amplified fragment migrates to 291 base pairs (bp). Figure 1 shows the electrophoretic migration to 291 bp in many investigated samples. An 100 bp molecular weight marker was used. For the amplification, a vaccine virus was used as positive control and ultrapure water as negative control. They were identified in the electrophoretic image with M + = positive control, and M - = negative control, being viewed in Figure 1 in the last two positions from left to right. All samples were operated in duplicate to ensure reproducibility and repeatability.

At position number 1, sample 1 is viewed as an intensely positive sample. Sample 1 comes from a 63-day-old male. Sample number 2 from the second position in the electrophoretic image is a weak positive sample, the electrophoretic lane being less visible. This indicates a smaller amount of amplicon/virus. Sample number 2 also belong to a 63-day-old male.

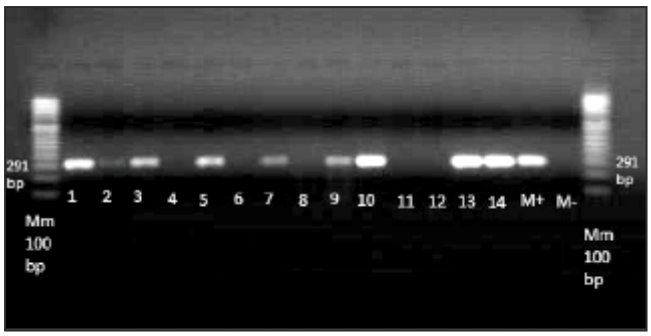

Fig. 1. Electrophoretic image with test results. Line Mm 100 bp= molecular weight marker of 100 base pairs; Line 1 = high positive sample; Line 2 = weak positive sample; Line 3 = positive sample; Line 4 = negative sample; Line 5 = positive sample; Line 6 = negative sample; Line 7 = weak positive sample; Line 8 = negative sample; Line 9 = positive sample; Line 10 = high positive sample; Line 11 = negative sample; Line 12 = negative sample; Line 13 = high positive sample; Line 14 = high positive sample; Line M+ = positive control; Line — = negative control. Positive samples for PRRS virus migrates in concordance with the molecular weight of 291 pairs of bases.

At position 3 is the result of sample number 3, being positive for the presence of PRRS virus. This sample comes from a male 60-day-old. Sample 5 is a positive one and belong to a 63-day-old female. At position 7, sample 7, is a weak positive sample, with a poor viral load, from a 60-day-old male, was observed. Sample 9 can be seen in Figure 1 at position 9 and is a positive sample also from a 60-day-old male. Sample number 10 described at position 10 is an intensely positive sample with a high viral load, from a 60 day-old female. Sample 13 from position 13 is highly positive, coming from a 120-day female, as well as sample 14 as equally positive, from a 130-day female. Samples 4, 6, 8, 11 and 12 are negative samples for the pre-sence of PRRS because no lanes are seen and come from a 60-day-old male (sample 4), 60-day-old female (sample 6), 60-day-old male (sample 8), 110-day-old female (sample 11), and 120-day-old male (sample 12).

In this study, in which 14 samples of EDTA blood collected were tested by classical PCR for the detection of PRRS virus, resulted are: 64.29% -positive samples and 35.71 % - negative samples.

Samples 1, 2, 3, 5, 7, 9, 10, 13, 14 were positive by the classical PCR method for the presence of PRRS virus. Samples 4, 6, 8, 11, 12 were negative for the PRRS virus. Positive samples came from individuals aged 60 days, 63 days, 120 days and 130 days of both males and females, namely 4 females and 5 males. Negative samples came from individuals aged 60 days, 110 days and 120 days, 2 females and 3 males.

The results obtained are complying with the respiratory clinical signs manifested by the pigs. The frequency of the positive samples is higher (64.29%) than the negative ones (35.71%).

DISCUSSIONS

These results confirm the presence of PRRSV in the farm, overlapping the prevalence of the respiratory diseases noticed, diseases representing the main pathology in the herd, blood being sampled in a relevant manner to the aetiology of clinical features expressed. The presence of the virus in this pig population it is to be expected, considering the lack of a prevention strategy for PRRS virus infection, both from the outside of the farm (the piglets origin farm) and within the farm. The specific and most recommended prophylactic action in this case is vaccination.

The study is further proof of the presence of Porcine Respiratory and Reproductive Syndrome on the territory of Romania. This has brought into discussion, through research, various strategies for monitoring, controlling and/or characterizing the genomic virus at farm level throughout the country. In recent years, many PRRS outbreaks have been reported across the country (12). In one of the studies conducted in South Eastern Romania, the clinical and serological examinations of sows (101-120 days) confirmed the disease, but epidemiological investigations did not lead to the identification of the source of the PRRS virus. This is also in line with the research of the present study, where there have been positive results in 120-day-old females (13).

Another study based on samples of randomly collected tissue from different pig farms in Romania aimed the assessment of the sensitivity and specificity of the RT-PCR method in virus detection and evaluation of the pathogenic virus charge in cases with clinical signs of PRRS. Samples were collected in farms from Brăila, Cluj, Arad, Iaşi and Satu Mare (14). In this study, conducted by Zăuleţ et al (2014), the fragment researched by PCR was the same as in the present paper, electrophoretic lanes migrating to 291 bp, and the positive samples were from Brăila, Cluj, Arad, Iaşi and Satu Mare County. This shows that the PRRS virus is still present in Braila County in swine populations.

As with the present study, previous research demonstrated the presence of the virus based on the ORF 7 sequence. At this point, it is necessary to continue monitoring the PRRSV in Romanian farms and to conduct further tests to genetically characterize the Romanian strains based on other genes, such as ORF 5, and to develop possible vaccines.

CONCLUSIONS

In this farm, the swine-hybrids are contaminated with the PRRS virus. The clinical features of PRRSV infection are those of the respiratory syndrome. The prevalence of infection in the sampled herd, in that series, was found to be higher in the age group of 60 days. Relating to the frequency of the infection in the investigated farm, the only control strategy to advice in order to improve the economic performance, is the vaccination.

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