

*Research article***SURVEY OF INFECTIOUS AGENTS ASSOCIATED WITH PORCINE RESPIRATORY DISEASE COMPLEX (PRDC) IN SERBIAN SWINE HERDS USING POLYMERASE CHAIN REACTION (PCR) DETECTION**

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A retrospective study on 235 natural cases of Porcine Respiratory Disease Complex in order to determine the etiological agents, their prevalence and interrelationships was performed in Serbia. Lung tissue samples were analyzed by Polymerase Chain Reaction for the presence of *Porcine circovirus type 2*, *Porcine reproductive and respiratory virus*, *Swine influenza virus*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Streptococcus suis* and *Arcanobacterium pyogenes*. A total of 49 different combinations of viral and bacterial pathogens were found. Five different viral and viral/Mhp co-infections were detected. Monobacterial infections were found in 150 cases and polybacterial infection was detected in 85 samples. PCV2 was the main virus detected, and Pm was the most aggressive secondary pathogen detected in PRDC. The reason for PRDC being so prevalent among Serbian pigs is most likely due to the large number of risk factors in the conventional farrow-to-finish system, compared to multi-site production systems. Therefore, measures aimed at a better control of respiratory viruses, particularly *Porcine circovirus type 2* and *Porcine reproductive and respiratory virus*, as well as *Mycoplasma hyopneumoniae* infections, and adoption of rational decisions on respiratory bacterial pathogens specific therapeutic and preventive strategies at herd level, simultaneously with significant improvements on farm management should reduce the occurrence of PRDC.

Key words: farm pigs, respiratory diseases, etiological agents, PRDC, PCR

INTRODUCTION

Porcine respiratory disease complex (PRDC) is a common term for pneumonia in finishing or fattening pigs caused by a multifactorial etiology [1,2]. A large number of

etiological factors are involved in the pathogenesis of PRDC, of which infective agents have the leading role, and they can be divided into four groups namely: viral respiratory pathogens (*Porcine Reproductive and Respiratory syndrome virus* (PRRSV), *Porcine circovirus type 2* (PCV2), *Porcine Cytomegalo Virus* (PCMV) and *Porcine Respiratory Corona Virus* (PRCV)); primary bacterial respiratory pathogens (*Mycoplasma hyopneumoniae* (Mhp), *Actinobacillus pleuropneumoniae* (App) and *Bordetella bronchiseptica* (Bord)); opportunistic bacterial respiratory pathogens (*Pasteurella multocida* (Pm), *Streptococcus suis* (Ssuis) and *Haemophilus parasuis* (Hps)); septicemic causes of pneumonia (*Salmonella enterica* (Salm), *Actinobacillus suis* (Asuis) and *Arcanobacterium pyogenes* (Apyo)) [1,2]. The etiology of PRDC varies between and within production herds and/or systems, however, the combination of primary and opportunistic infectious agents is generally involved [3]. Combinations of viruses and/or Mhp and opportunistic bacteria have been reported to work in a synergistic fashion producing more severe and/or fatal pneumonias than those induced by each individual agent [4]. The most common respiratory pathogens reported are Mhp, PRRSV, PCV2, Pm, App, Hps and Sspp [5-8]. PRDC is frequent and usually present in farrow-to-finish systems in Serbia, however, a comprehensive survey of the pathogens involved in PRDC in Serbian pigs has never been performed. In recent years the considerable genetic diversity of some respiratory pathogens has been described [9,10] and a survey of respiratory pathogens in pigs has become relevant. Therefore, the objectives of this investigation were to detect the viral and bacterial pathogens, to determine the prevalence, and interrelationships of the detected microorganisms in the lung lesions of pigs which died from PRDC, originating from ten conventional farrow-to-finish pig farms chosen as representative of the Serbian swine industry.

MATERIAL AND METHODS

The survey was carried out from September 2011 to August 2012 on ten pig farms. The studied farms were located in the north (Vojvodina province, 8 farms) and central part of Serbia (central Serbia, 2 farms) where approximately 85% of Serbian market pigs are produced. The farms in the study were all single site farrow-to-finish, with a 1200 to 1500 sows per farm, producing about 8% of market pigs in Serbia. All farms practiced continuous flow operations, and had poor management of health issues including: biosecurity, strategy to minimize disease challenge, inadequate sanitation, room management and vaccination scheme, as well as housing-environmental-management shortcomings. All farms included in the study had never implemented vaccination against PRRSV, PCV2, and only two farms had vaccinated against App, Pm and Hps previously. On four farms, vaccination against Mhp was irregularly applied. Respiratory diseases, were the most important cause of death in weaned and growing-finishing pigs. Overall mortality from wean-to-finish, during the period from the end of October 2011 to late-April 2012, reached 22-40%, whilst the mortality rates due to respiratory disease ranged from 20-37%. Thereafter, the total mortality (from

wean-to-finish) was gradually decreased, and at the end of the survey (August 2012), the mortality rate due to respiratory diseases, ranged from 9-15%. Each farm was visited once, at the mortality peak (20-37%), in weaned and grow-finishing pigs (from November 2011 to March 2012), for observation and collection of suspected material for further investigation. On necropsy, pneumonic lungs from at least 20 of the dead, weaned (8-12 weeks of age) and growing-finishing (12-25 weeks of age) pigs, were collected for laboratory testing. Macroscopic lung lesions which were most frequently observed included: (a) bronchointerstitial pneumonia, (b) pleuropneumonia, (c) suppurative pneumonia and (d) pneumonia with abscesses, respectively. Resection of the trachea up to the bifurcation was performed and sterile PBS was administered into both bronchi. Samples were taken with cotton swabs by immersion into the contents from the caudal lung lobe and by swabbing the mucosa of the bronchus, trachea and lesions. Swab tubes were additionally filled with 2 ml of sterile phosphate buffered saline (PBS).

In the laboratory, 1 ml of suspension from each tube was centrifuged at 14,000 g (Hettich Micro 20) for 10 min, washed in sterile PBS and resuspended in 180 µl of ATL buffer. The samples were incubated in 400 µl proteinase K lysis buffer for 1 h at 60 °C. The lysed samples were processed with a DNA extraction kit (QIA extraction DNA kit, Qiagen) according to the manufacturer's instructions. All samples were tested by PCR using HotStarTaq Master Mix Kit (Qiagen), and primers described for DNA detection of PCV2 [11], Mhp [12], Pm [13], App [14], Hps [15], Ssuis [16] and Apyo [17]. After suspension, the samples were processed with a RNA extraction kit (Qiagen – Rneasy MiniKit) according to the manufacturer's instructions. For detection of PRRSV, RT-nested PCR (OneStep RT-PCR Kit; HotStarTaq Master Mix Kit - Qiagen) specific for ORF 7 [18], and for *Swine Influenza Virus* (SIV) detection, RT-PCR (OneStep RT-PCR Kit – Qiagen) specific for type A influenza virus were used [19].

To determine, whether the viral, and/or the viral/Mhp co-infections which were detected varied in prevalence, whether there were significant differences between the prevalence of mixed infections, and to assess the significance of the association among viral and/or viral/Mhp co-infections, with Pm, App, Hps, Ssuis and Apyo, contingency tables and Chi-squared tests were used for statistical analyses.

RESULTS

In total, DNA target sequences from PCV2, Mhp, PRRSV, SIV, Pm, App, Hps, Ssuis and Apyo were detected in 207, 185, 141, 4, 137, 87, 76, 33 and 10 of the samples, respectively. Of the 235 samples examined, PCV2/Mhp, PCV2/PRRSV/Mhp, PCV2/PRRSV, PCV2/PRRSV/SIV/Mhp and PRRSV/Mhp co-infections, in combination with other bacterial respiratory pathogens were detected in 94 (40%), 59 (25%), 50 (21.2%), 28 (11.9%), and 4 (1.7%) of the samples, respectively, (Table 1). Along with these five viral, and viral/Mhp co-infections detected, DNA, from Pm (57%), App (37%), Hps (32.3%), Ssuis (14%) and Apyo (4.2%) was also detected

in the samples examined. Mono bacterial infections were detected in 150 (63.8%) samples whereas 85 (36.1%) samples yielded poly-bacterial infections (Table 2). A total of 49 different combinations of viral and bacterial pathogens were found in PRDC cases examined. However, mixed infection where three agents were involved was the most frequently detected pattern (116/235), followed by situations where 4 (79/235), 5 (27/235), 6 (11/235) and 7 (2/235) agents were detected respectively (Table 1). Statistically, these differences were significant ($p < 0.01$), and indicating that in pigs which are suffering from PRDC, there is almost a 50% possibility that at least three agents, are most frequently involved in the pathogenesis of the disease. The most frequently detected 3-agent combination was PCV2/Mhp/Pm (26/116 cases). The most common 4-agent combination cases were PCV2/PRRSV/Mhp/Pm (12/79). Five agent combination cases comprising PCV2/PRRSV/Mhp/Pm/APP (8/27) were seen most often, while when six agents combination cases were found, PCV2/PRRSV/Mhp/Pm/APP/Hps (4/11) were the pathogens found most often. Seven agents mixed infections (2/235) were rare but the two cases found had the following pathogens present PCV2/PRRSV/Mhp/Pm/APP/Hps/Ssuis and PCV2/PRRSV/Mhp/Pm/APP/Hps/Apyo. However, the PCV2/Mhp co-infection (94/235), was detected significantly more ($p < 0.01$), than other viral, and/or viral/Mhp co-infections, thus implicating PCV2 and Mhp, as the two most common agents, in PRDC cases (Table 2). Nevertheless, together, the others viral, and/or viral/Mhp co-infections (141/235), were significantly more often detected ($p < 0.05$) compared to the PCV2/Mhp co-infection (94/235).

In addition to Mhp, the highest detected bacterial pathogen in examined PRDC cases

Table 1.

Agents	n=samples	%	mixed infection	Total	%
PCV2+Mh	94	40	3-agent mixed inf.	116	49.3
PCV2+PRRS+Mh	59	25	4-agent mixed inf.	79	33.6
PCV2+PRRS	50	21.2	5-agent mixed inf.	27	11.4
PRRS+Mh	28	11.9	6-agent mixed inf.	11	4.6
PCV2+PRRS+SIV+Mh	4	1.7	7-agent mixed inf.	2	0.8
TOTAL	235	100	5	235	100

Viral and viral/Mhp co-infections (the first three columns), as well as mixed (viral/bacterial) infections (the last three columns) in which three, four, five, six or seven agents were detected by PCR, in lung tissue samples of 235 PRDC cases studied, originating from ten conventional farrow-to-finish pig farms in Serbia.

was Pm (137/235), which is significantly associated ($p < 0.01$) with all viral, and/or viral/Mhp co-infections detected, as well as with other opportunistic bacterial respiratory pathogens, App and Apyo. The App was significantly more ($p < 0.01$) detected with the PCV2/PRRSV (15/50) and PRRS/Mhp co-infections (13/28) in examined PRDC

Table 2.

	Pm	APP	Hps	Ssuis	TOTAL	Pm + APP	Pm + Hps	Pm + Ssuis	Pm + Appo	APP + Hps	APP + Appo	Pm + APP	Pm + Hps	Pm + Ssuis	Pm + Appo	APP + Hps	APP + Appo	TOTAL
PCV2+Mh	26	14	16	4	60	12	7	4	2	3	-	5	-	1	-	-	-	34
PCV2+PRRS	19	7	5	7	38	5	3	-	1	1	-	2	-	-	-	-	-	12
PCV2+PRRS+Mh	7	8	9	8	32	8	6	1	-	1	-	4	1	1	2	2	1	27
PCV2+PRRS+SIV+Mh	1	-	-	1	2	1	-	-	-	-	-	-	-	1	-	-	-	2
PRRS+Mh	6	8	2	2	18	2	2	2	1	1	1	1	-	-	-	-	-	10
TOTAL	59	37	32	22	150	28	18	7	4	6	1	12	1	1	4	2	1	85

Viral and bacterial pathogens detected by PCR, in lung tissue samples of 235 PRDC cases analyzed, in the ten selected farrow-to-finish pig farms from Serbia.

cases. The Hps is significantly more associated ($p < 0.01$) with the PRRSV/Mhp and PCV2/Mhp co-infections, while the Ssuis is significantly associated ($p < 0.01$) with PCV2/PRRSV/Mhp co-infection.

Finally, the Apyo can be found in samples with all viral, and/or viral/Mhp co-infections, in studied PRDC cases.

It was not possible to compare the association of SIV with other pathogens since it was detected only in a few samples.

DISCUSSION

The results of this survey provide a further insight into the prevalence and diversity of major infectious agents, and their interrelationships in PRDC. However, the most significant conclusion indicates that PCV2, Mhp, PRRSV and Pm were the most frequently present pathogens in PRDC in studied the pigs. Moreover, the frequency of occurrence of certain pathogen profiles in the cases examined allows the prediction of the most common infectious patterns which lead to the emergence of PRDC.

The here obtained results show that PCV2 is the most prevalent pathogen detected in the majority of lung samples examined although using PCR as a method for PCV2 identification may be inconclusive on the importance of this virus for the induction of pathological lesions in the lungs. Accordingly, the PRDC cases we examined can be seen as field models, demonstrating that PCV2 replication and associated lesions can be enhanced by concurrent infection with other viruses or bacteria though the exact mechanisms by which concurrent pathogens upregulate PCV2 are unknown [20]. Interestingly if PCV2 is excluded from any pathogen combination, detected in this study, PRDC remains the most common mixed respiratory infection (141/235), compared with “classical” enzootic pneumonia which is significantly less present (94/235), suggesting that PRDC is the most prevalent respiratory disease in the swine population. Therefore, these findings are in accordance with other reports on the high prevalence of PRDC, and the significant association of PCV2 in PRDC in naturally infected pigs using both, PCR [6] and in situ hybridization (ISH) detection of PCV2 [21].

The high prevalence of PRRSV detected in the PRDC cases in this study strongly supports an important role of PRRSV in PRDC and implicates PRRSV as an important viral respiratory agent in the Serbian swine herds. In addition, the high detection rate of Mhp in the studied PRDC cases is in line with the results of other reports on the role of Mhp as a primary bacterial respiratory pathogen in this complex, preparing the way for others to produce more severe lesions [3,7].

Pasteurella multocida was significantly associated with all viral, and/or viral/Mhp combinations detected in this study, as well as with other opportunistic bacteria. This confirms that Pm most likely does not act as a primary pathogen but rather follows infections with other agents. It does significantly aggravates the lung lesions in PRDC

which have experienced earlier infections by immunosuppressive viruses and Mhp [22].

As App is a primary bacterial respiratory pathogen, able to cause the disease on its own, the high co-occurrence of App and viral or viral/Mhp co-infections in PRDC cases from this study is probably coincidental, however, its participation in the PRDC is significant compared with some other field studies [5,6] indicating a high prevalence of App in the Serbian swine population. This suggests high infection pressure, probably generated from the continuous operation flow and mixing of the different age groups of pigs that share the same air space.

Haemophilus parasuis is considered as a common inhabitant of the upper respiratory tract of asymptomatic pigs raised under commercial conditions and as one of the opportunistic bacterial respiratory pathogens in PRDC [23]. Thus, relatively high prevalence of Hps in the polybacterial lesions in this PRDC study (one-third of the samples were positive for Hps) may be attributed to the simultaneous immunosuppressive effects of the viruses and Mhp, which caused an increased number of the bacteria including the Hps in the lungs. Furthermore, in Hps-monobacterial infections (32/235), the most probable cause of pig death was septic shock emerging as a result of rapid proliferation of Hps in the immunocompromised lungs [24].

Since the role of Ssuis as a primary agent in pulmonary lesions in the absence of other pathogens is still controversial [1], in the PRDC, it was recognized as an opportunistic bacterial pathogen. Therefore, septicemia is the most likely reason, for a significantly higher detection of this pathogen in mono, than in mixed bacterial infections of PRDC samples from our study (22/32).

Arcanobacterium pyogenes is another common pathogen on the mucous membranes of the upper respiratory tract [25]. However, devitalized or inflamed tissue is a prerequisite for Apyo to cause suppurative inflammation and abscessation, therefore in the lungs, it acts as a secondary invader in a pre-existing pneumonia. Many pathogens including PRRSV and Pm may damage the upper respiratory tract epithelium [26] creating a portal for Apyo. Nevertheless, Apyo is not a typical respiratory pathogen and its detection with accompanying lesions in the lungs from the PRDC cases in this study may be illustrative of the extraordinary versatility of Apyo as a pathogen.

Our study confirms that PRDC involves multiple pathogens and that the microorganisms most commonly associated with the PRDC are present and widespread in conventional farrow-to-finish systems. The previously reported occurrence of corresponding pathogen profiles in PRDC is confirmed by the results of the present study. PCV2 appears to be the major viral pathogen involved in PRDC, however its role is likely to be centered on the interaction with other pathogens. PRRSV was the next most common virus participating in PRDC, and although there were too few positive SIV samples, it does indicate that SIV is circulating in the Serbian swine population. Beside

Mhp, the Pm was the individually the highest detected bacteria, indicating Pm primarily, as the most aggressive secondary pathogen in the PRDC cases studied.

Respiratory diseases among Serbian pigs seem to currently tend toward PRDC definition, compared to enzootic pneumonia. Explanation for this can be both, the introduction of sophisticated methods for detection of respiratory pathogens, particularly for PCV2, and Mhp detection, and the presence of a larger number of risk factors for respiratory diseases occurrence in conventional farrow-to-finish systems. The latter can provide more opportunities for multiple interactions between pathogens, compared to multi-site production systems.

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IDENTIFIKACIJA INFEKTIVNIH AGENASA U KOMPLEKSU RESPIRATORNE BOLESTI SVINJA (PRDC) NA FARMAMA SVINJA U SRBIJI PRIMENOM REAKCIJE LANČANE POLIMERAZE (PCR)

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U cilju identifikacije, utvrđivanja prevalence i interakcije multiplih infektivnih agenasa u kompleksu respiratorne bolesti svinja (PRDC), retrospektivno je pregledano 235 uzoraka tkiva pluća poreklom od prirodno inficiranih svinja. Uzorci su analizirani reakcijom lančane polimeraze (PCR) na prisustvo *Porcine circovirus tip 2* (PCV2), Virus reproduktivnog i respiratornog sindroma svinja (PRRSV), Virus influence svinja (SIV), *Mycoplasma hyopneumoniae* (Mhp), *Pasteurella multocida* (Pm), *Actinobacillus pleuropneumoniae* (App), *Haemophilus parasuis* (Hps), *Streptococcus suis* (Ssuis) i *Arcanobacterium pyogenes* (Apyo). U ispitivanim uzorcima, ukupno je ustanovljeno 49 različitih kombinacija virusnih i bakterijskih patogena. Koinfekcije virusnih i/ili virusnih i Mhp infekcija sa odgovarajućim bakterijskim patogenima su ustanovljene u pet različitih kombinacija. Mono bakterijske infekcije su detektovane u 150, a poli-bakterijske infekcije su ustanovljene u 85 ispitivanih uzoraka. PCV2 je najfrekventnije detektovani virus, a Pm je najznačajniji sekundarni bakterijski respiratorni patogen ustanovljen u ispitivanim PRDC slučajevima. Razlog zbog koga je PRDC trenutno najfrekventnije ustanovljena respiratorna afekcija svinja u Srbiji je najverovatno zbog prisustva većeg broja fakotrizika u konvencionalnim zatvorenim sistemima gajenja svinja.