









High prevalence of porcine circovirus 2, porcine parvovirus, and pathogenic leptospires in mummified swine fetuses in Southern Brazil

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ABSTRACT: Modern swine production employs a high degree of technology and is organized in various production stages, in which reproduction is one of the most significant. However, reproductive losses associated to fetal death are still high. Fetal losses, including fetal mummification, may occur at a rate below 1.5% in a sound herd. The causes of fetal death can be very diverse, and include various infectious agents. Therefore, investigating the causes of fetal losses is important in the decision-making process related to the control and prophylaxis of the herd. The goal of this study was to carry out a molecular survey, analyzing porcine circovirus 2 (PCV2), porcine parvovirus (PPV), and pathogenic *Leptospira* as potential agents related to fetal death in swine farms in Southern Brazil. We collected samples from three farms with a mummified index $\geq 2.5\%$. Fragments of brain, lung, kidney, liver, and heart were sampled for PCR analysis. Out of 100 samples examined, 87, 68, and 22 were positive for PCV2, PPV, and *Leptospira* spp., respectively. Moreover, we also identified coinfections with two and three pathogens in the same sample. Our findings contribute to the spreading of scientific knowledge related to infectious causes (PPV, PCV2, and pathogenic leptospires) of fetal losses in swine, as evidenced by the high frequencies of the investigated agents.

Key words: fetal losses, leptospire, PCV2, PPV, reproductive failure.

Alta prevalência de parvovírus suíno, circovírus suíno 2 e leptospiros patogênicas em fetos suínos mumificados na região Sul do Brasil

RESUMO: A produção moderna de suínos emprega um alto grau de tecnologia e é organizada em várias etapas de produção, nas quais a reprodução é uma das mais importantes. No entanto, as perdas reprodutivas associadas à morte fetal ainda são altas. Perdas fetais, incluindo mumificação, podem ocorrer em taxa abaixo de 1,5% em rebanhos sadios. As causas da morte fetal podem ser diversas, incluindo causas infecciosas. Portanto, a investigação de causas relacionadas à ocorrência de perdas fetais é importante no processo relacionado ao controle e profilaxia do rebanho. O objetivo deste estudo foi realizar um levantamento molecular, incluindo circovírus suíno 2 (PCV2), parvovírus suíno (PPV), e leptospiros patogênicas como potenciais agentes relacionados à morte fetal em suínos no sul do Brasil. Foram coletadas amostras de três granjas com índice de fetos mumificados $\geq 2,5\%$. Fragmentos de cérebro, pulmão, rim, fígado e coração foram amostrados para análise por PCR. Das 100 amostras examinadas, 87 foram positivas para PCV2, 68 foram positivas para PPV e 22 foram positivas para *Leptospira* spp. Além disso, também identificamos co-infecções com dois e três patógenos em uma mesma amostra. Os resultados encontrados contribuem para a disseminação do conhecimento científico relacionado às causas infecciosas (PCV2, PPV e *Leptospiros patogênicas*) relacionadas às perdas fetais em suínos, assim como evidenciam a alta frequência dos agentes pesquisados.

Palavras-chave: falhas reprodutivas, *Leptospiros*, PCV2, perdas fetais, PPV.

Brazil is one of the most important meat producers and exporters worldwide, and its Southern region accounts for approximately 84% of all Brazilian pork exportation (ABPA, 2018). The dimensions of this pork production chain inevitably call for a stringent set of approaches towards animal health and reproductive performance (VARGAS et al., 2007). In that regard, fetal mummification has a negative impact on the total number of piglets born alive per litter (BORGES et al., 2005). According

to ZANELLA, SILVEIRA & SOBESTIANSKY (2007), fetal mummification rates of up to 1.5% are generally acceptable; however, there is an increasing number of reports indicating a steep increase in fetal mummification, with rates of over 3% being reported (data not published). Fetal mummification in swine is associated with a wide range of factors, including lack of uterine space, exposure to toxic substances, and infectious diseases (ZANELLA; SILVEIRA & SOBESTIANSKY, 2007).

Infections caused by porcine circovirus 2 (PCV2), porcine parvovirus (PPV), and *Leptospira* spp. (MORÉS; BARCELLOS & ZANELLA, 2007) are among the most important causes of reproductive failure. Porcine circovirus type 2 is mainly linked to post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS), as well as reproductive disorders, including fetal mummification (ASANOME et al., 2008; MAES et al., 2008; MADSON et al., 2009).

Porcine parvovirus is the main infectious cause of fetal mummification in swine (ROEHE; SOBESTIANSKY & BARCELLOS, 2007) and has been associated with reproductive failure in Brazilian swine farms for about five decades (GOUVÊIA et al., 1984). This virus infects tissues with high rates of mitosis, such as the fetus and placenta (ROEHE; SOBESTIANSKY & BARCELLOS, 2007), and causes embryo resorption, delayed parturition, mummified fetuses, stillbirths, and perinatal death (PASCOAL et al., 2006).

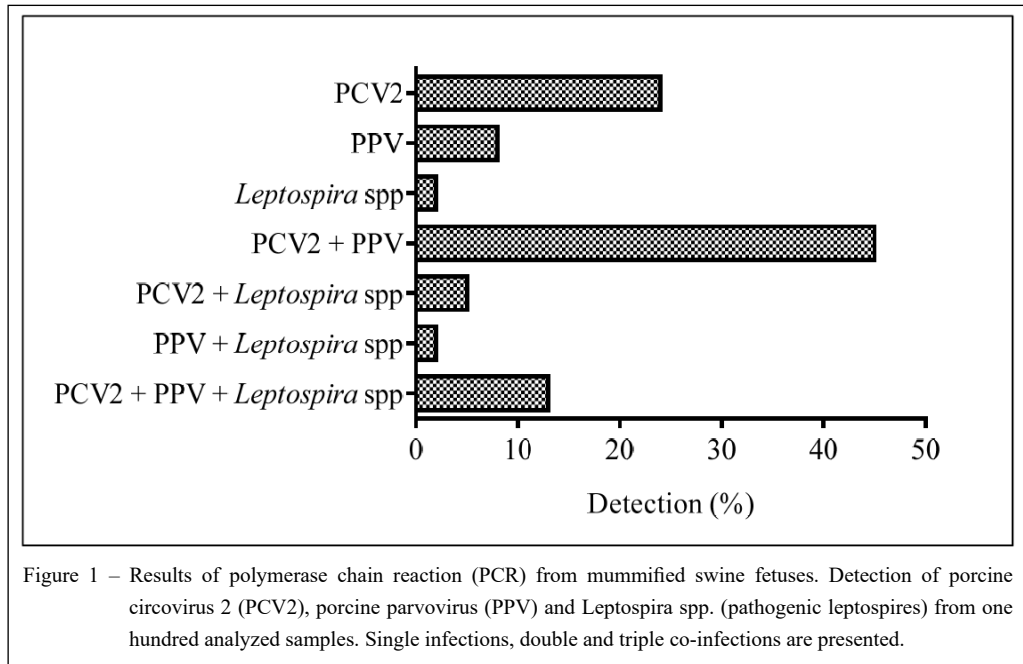
Leptospirosis is a zoonosis and a widespread occupational hazard, globally associated with significant losses in porcine reproduction. It is especially important in Southern and Southeastern Brazil (LANGONI et al., 1995). Prevalent serovars, for instance Pomona, Icterohaemorrhagiae, Tarassovi, Canicola, and Bratislava (SOBESTIANSKY et al., 1999) cause embryo loss, anestrus, abortion, dystocia, reduced litters, fetal mummification, stillbirths, and runt piglets (EDWARDS, 1979). The bacteria may persist in the kidney and genital tissues for up to five months after an abortion (ELLIS & THIERMANN, 1986).

Given the importance of these agents in swine reproduction, the goal of this study was to investigate whether PCV2, PPV, and leptospires were involved in the increasing numbers of fetal mummification in the pig farms of Southern Brazil. Samples were collected from three pig farms in the southernmost Brazilian states (Paraná, Santa Catarina, and Rio Grande do Sul) with reported occurrences of mummification $\geq 2.5\%$. At least 30 females with different parity orders and two or more mummified fetuses from each origin were considered for this study. Fragments of brain, lung, kidney, liver and heart, and liver were collected and pooled from fetuses collected immediately after parturition and stored at $-20\text{ }^{\circ}\text{C}$. The isolation and purification of total DNA (50 – 100 mg in 10% w/v PBS) was achieved using silica-based spin columns according to the manufacturer's instructions (BIOPUR Kit de Extração Mini Spin Plus, Biometrix Diagnóstica Ltda., Curitiba, PR, Brasil). The PCR detection of the infectious agents was based

on the amplification of conserved sequences of genes that codify for the capsid protein (*ORF2*) of PCV2 (RINCÓN MONROY et al., 2014) and for the non-structural protein (*NSI*) of PPV (SOARES et al., 1999). Leptospires were detected through amplification of the genes *LigA* and *LigB* (PALANIAPPAN et al., 2005), expressed as conserved virulence factors reported only in pathogenic bacteria (MATSUNAGA et al., 2003). Polymerase chain reactions for PCV2, PPV, and *Lig* genes amplified fragments of 657, 330, and 468 base pairs, respectively, using a ready-to-use premixed solution (GoTaq Green Master Mix, Promega), plus 500 nM of primers, 2 μL of template DNA, and ultrapure nuclease-free water to a final reaction volume of 25 μL . In each PCR run, a non-template control was included to check for contamination. In addition, positive controls (DNA samples isolated from known positive tissues) were run in parallel with unknown samples to ensure proper assay quality. Amplified products were resolved by electrophoresis on a 1% agarose gel and visualized using an intercalating stain (Unisafe Dye, Uniscience do Brasil, SP, Brazil) in a high-resolution/sensitivity imaging system (ChemiDoc Gel Imaging System, BioRad, EUA).

A total of 100 samples were tested for the presence of PCV2, PPV, and *Leptospira* spp. Eighty seven percent were positive for PCV2 (87/100), 68% were positive for PPV (68/100), and 22% were positive for pathogenic leptospires (22/100). Infection by a single agent totaled 24 samples positive for PCV2, eight samples positive for PPV, and two samples positive for *Leptospira* spp. Coinfection with PCV2/PPV, PCV2/*Leptospira* spp., and PPV/*Leptospira* spp. occurred in 45, 5 and 2 samples, respectively. Additionally, 13 samples were positive for all three agents investigated in this study (Figure 1).

Due to their variety, PCV2-related diseases have been extensively studied (ZANELLA; SILVEIRA & SOBESTIANSKY, 2007). However, there are only a handful of studies suggesting the involvement of PCV2 in porcine reproductive failure. A Canadian report published in 1999 was the first to associate PCV2 with reproductive failure (WEST et al., 1999). ROCHA et al. (2010) reported a study in which 50.3% of samples were positive for PCV2 (stillbirths and mummified fetuses). Our results clearly indicated a high percentage of mummified fetuses positive for PCV2 (87%). Swine populations with a high frequency of PCV2 have frequently been start-up herds with a significantly high number of susceptible gilts (YU et al., 2007). Even though vaccines against PCV2 infection are considered effective (OPRIESSNIG et al., 2008), it



might be necessary to readdress vaccination protocols regarding the time point of dam immunization, which can influence the level of circulating antibodies (AFGHAH et al., 2017). OLIVER-FERRANDO et al. (2018) detected a subclinical infection in seropositive gilts and sows along with a low percentage of viremic animals before starting vaccination trials. Vaccination protocols are not yet fully standardized in Brazil, so that the maintenance of the virus in farms might be facilitated. However, it is important to emphasize that immunization without the appropriate farm management or biosafety measures does not prevent PCV2-related problems.

Our findings indicated that the molecular detection of PPV was substantial. Conversely, PESCADOR et al. (2007) reported that 2.4% of stillbirths and mummified fetuses were PPV-positive in Southern Brazilian farms, while MALDONADO et al. (2005) did not detect the presence of PPV in stillborn and aborted fetuses in Spain. This agent is ubiquitous in swine populations worldwide (SHARMA & SAIKUMAR, 2010). Thus, widespread vaccination against PPV is regarded as an important tool to control reproductive problems (MALDONADO et al., 2005; SHARMA & SAIKUMAR, 2010). Interestingly, our findings indicated a high frequency of PPV in mummified fetuses despite vaccination. It is noteworthy that we observed coinfection with PCV2 and PPV (45/100

samples). Coinfection with PCV2 and PPV has been associated with PMWS and abortion in naturally and experimentally infected pigs (KENNEDY et al., 2000). The PPV replicates in cells of the monocyte-macrophage series and may produce immune cell dysfunction, activation, or immunosuppression, enhancing the replication of PCV2 (KENNEDY et al., 2000). However, it is not clear whether PCV2 alone is causing the reproductive failure or a coinfection with PPV could trigger these pregnancy disorders. Few vaccination protocols in Brazil use the PCV2 vaccine to prevent pregnancy losses. Thus, it might be possible that reproductive failures associated with return to estrus, early abortion, and fetal mummification have been incorrectly related to other agents or non-infectious causes. The high rate of PCV2 infection reported in our study suggested that PCV2 vaccination before breeding could be used as an important tool to decrease reproductive issues.

There are a great variety of *Leptospira* serovars present in swine herds worldwide, causing stillbirths, mummified fetuses, and the births of runt piglets (RAMOS et al., 2006). Fortunately, only a small number of serovars are endemic to any particular region or country, so that the clinical signs associated with leptospiral infection often include correlations with serovars. Our study did not accomplish a serovar differentiation, as we focused on the molecular detection of pathogenic leptospires.

Although, not as prevalent as PCV2 and PPV, we observed that 22% of samples were positive for *Leptospira* spp. The pathogenesis of the reproductive disease is still poorly understood. Nevertheless, some authors accept that the transplacental infection that occurs during a very transient maternal leptospiremia is the sole cause of disease development (ELLIS & THIERMANN, 1986). Despite the scarce molecular data on the detection of leptospirosis associated with reproductive issues in swine, our results are quite important. Even considering vaccination as one of most important tools to control leptospirosis in production animals (VALÉE et al., 2017), the efficacy of the swine vaccines is questionable, as they induce limited amounts of protective antibodies and are rarely produced with the strains that affect the herds (BALAKRISHNAN & ROY, 2014). SONADA et al. (2018) published an interesting report about the efficacy of the commercial vaccine against leptospirosis. They demonstrated that three out of the five vaccines sold in Brazil for the immunization of swine (or cattle) failed to protect the vaccinated animals following a challenge with an autochthonous *Leptospira* strain. These results might support our findings, for all samples analyzed in our study came from regularly vaccinated herds.

Based on our results, we were able to demonstrate high frequencies of PCV2 and PPV, as well as pathogenic leptospirems in mummified swine fetuses. These findings may explain the current increased rates of mummified swine fetuses observed in Southern Brazil, and indicate the need for further study on circumventing the reproductive issues caused by these agents.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The authors declared, for all due purposes, the project that gave rise to the present data does not require IACUC or CEUA (Institutional Animal Care and Use Committee; Comissão de Ética no Uso de Animais) approval because it did not use LIVE ANIMALS. Thus, the authors assume full responsibility for the presented data and are available for possible questions, should they be required by the competent authorities.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest. The funding sponsors had no role in the design of the study; collection, analyses, or interpretation of data; writing of the manuscript; or decision to publish the results.

AUTHORS' CONTRIBUTIONS

GH, AEM, SAM performed the experiments. AAT, LKG, SAM, PEB supervised and coordinated the lab analysis. MM, SAM and PEB analyzed the results and AAT, MM, SAM and PEB writing and critically revised the manuscript. DLV, DM, NCB, GH, and AEM processed the samples.

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