



Short communication

Pluck-pools as diagnostic samples for detecting porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 in porcine abortion material and stillbirths

Heinrich Kreutzmann^{a,*}, Christine Unterweger^a, Lukas Schwarz^a, Katharina Dimmel^b, Angelika Auer^b, Till Rümenapf^b, Andrea Ladinig^a

^a University Clinic for Swine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

^b Institute of Virology, Department for Pathobiology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

ARTICLE INFO

Keywords:

Pluck-pool
Porcine circovirus type 2
Porcine fetus
Porcine reproductive and respiratory syndrome virus
Reproductive failure

ABSTRACT

Investigating infectious agents in porcine abortion material and stillborn piglets poses challenges for practitioners and diagnostic laboratories. In this study, pooled samples of individual reference organs (thymus and heart) from a total of 1000 aborted fetuses and stillborn piglets were investigated using quantitative PCR protocols for porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) and porcine circovirus type 2 (PCV2). Simultaneously, a pluck-pool containing equivalent portions of fetal thymus, heart, and lung tissue was collected, frozen at -20°C , and re-analyzed when a certain amount of either PRRSV-1 RNA or PCV2 DNA was detected in individual reference organs. Thirteen pluck-pools were assessed for PRRSV-1, all being PCR-positive. For PCV2, 11 of 15 pluck-pools investigated were PCR-positive. In all pluck-pools testing negative, viral loads in individual pools were low. This study indicates that pluck-pools can be valuable diagnostic material and the consolidation of multiple organs through a single RNA/DNA extraction optimizes the utilization of available laboratory resources. Additional research is required to assess the feasibility of follow-up investigations and to accurately define criteria for interpretation of viral loads in a clinical context.

Infectious agents in pigs can cause reproductive failure either indirectly by systemic effects on the sow or directly by affecting the reproductive tract (Christianson, 1992). Viruses directly affecting fetuses such as porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) and porcine circovirus type 2 (PCV2), are commonly observed in Austrian farms, and since they are associated with fetal death, they are often the subject of diagnostic investigations (Reif et al., 2022; Sinn et al., 2016; Unterweger et al., 2021, 2023). Careful consideration needs to be given to the number of piglets to be examined, the choice of material to be tested, and the specific method to be used (Kreutzmann et al., 2022; Pensaert et al., 2004). For PRRSV-1 diagnosis, fetal thymus is recommended (Cheon and Chae, 2001), while for PCV2, the fetal heart is considered as the reference organ (Brunborg et al., 2007; Hansen et al., 2010). Hardly any information exists on the use of pooled samples from multiple organs of several fetuses. In this study, the detection of PRRSV-1 and PCV2 in pooled samples of individual reference organs was compared to the detection rate of both pathogens in pluck-pools

containing equivalent portions of fetal thymus, heart, and lung tissue.

This study included 1000 fetuses and stillborn piglets from 127 distinct farms across five different Austrian federal states. These fetuses and stillborns had been submitted to Vetmeduni Vienna for routine diagnostic purposes between 2019 and 2023. A total of 251 thymus-, heart-, and pluck-pools (thymus, heart, and lung tissue; each organ approximately 0.5 cm^3 in size) were collected. Each of the pooled samples consisted of tissues from usually more than one fetus or stillborn from the same litter, averaging 3.98 ± 1.85 individuals in each pool (Supplementary Table S1). A 10% tissue suspension was prepared by homogenizing 100 mg of tissue (containing equal sized pieces from all pooled tissues) with 1 mL sterile PBS in a microcentrifuge tube containing stainless steel beads using a TissueLyser II (Qiagen). Following centrifugation, 140 μL of supernatant was used for extraction. DNA as well as RNA were extracted simultaneously by employing the QIAamp Viral RNA Mini QIAcube Kit (Qiagen) according to the manufacturer's instructions. If a pool consisted of more than nine pieces of tissue, these

* Correspondence to: GD Animal Health Service, P.O. Box 9, 7400 AA Deventer, the Netherlands.

E-mail address: h.kreutzmann@gddiergezondheid.nl (H. Kreutzmann).

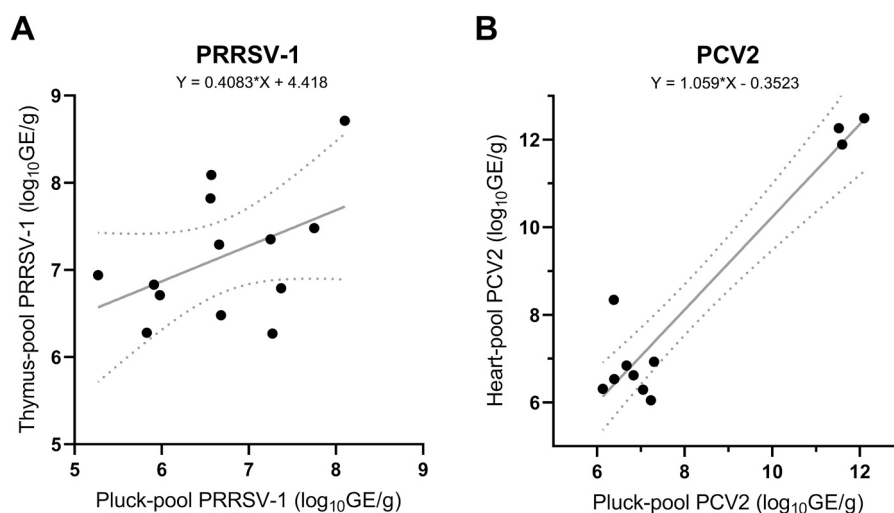


Fig. 1. Scatterplot of (A) porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) and (B) porcine circovirus type 2 (PCV2) positive samples. The viral loads in individual organ pools (thymus for PRRSV-1, heart for PCV2) are presented on the respective Y-axis, while the viral loads of the re-evaluated pluck-pools are depicted on the X-axis. The solid gray line was computed using simple linear regression, with the corresponding equation provided in the figure. The dotted lines illustrate the error bars. The simple linear regression equation and the error bars were calculated with GraphPad Prism 9.0.0 for Windows (GraphPad Software).

were weighed, supplemented with 10 times the amount of PBS and homogenized using a BagMixer (Interscience). Then 1 mL of the liquid was centrifuged and extracted as described above. For detection of PRRSV-1 specific nucleic acids, an in-house RT-qPCR targeting ORF1 (primer sense: 5'-TTTATTCTCGACTCCATCCAACC-3', primer antisense: 5'-AAAGTTGGCGCTGCTCA-3' and probe FAM-5'-TCTTCTTGTCGAS-CACGATTCGCCG-3'-BHQ1) was used with a cut-off set to 10^5 genome equivalents (GE)/g tissue. In total, 246 thymus-pools were examined, with 13 of them showing positive results (viral load range: 1.85×10^6 to 5.10×10^8 GE/g). For PCV2 detection, a qPCR was used with a cut-off set to 10^6 GE/g tissue (Hoffmann, 2016). In total, 239 heart-pools were investigated, with 15 yielding positive results (viral load range: 1.01×10^6 to 3.12×10^{12} GE/g).

Pluck-pool samples, frozen at -20°C , were analyzed retrospectively when thymus- and/or heart-pools were considered positive. Of the 13 pluck-pools investigated for PRRSV, all were PCR-positive. In addition, 11 of 15 pluck-pools investigated for PCV2 were PCR-positive. The four corresponding heart-pools of the negatively tested pluck-pool samples showed a viral load between 1.01×10^6 GE/g and 6.70×10^6 GE/g. These results are displayed as scatterplots in Fig. 1A and B, and the raw data is provided in Supplementary Table S1.

The linear regression curve depicted in Fig. 1A demonstrates lower PRRSV viral loads within the pluck-pools. This outcome is possibly due to a dilution effect, given that cardiac tissue is not usually associated with a high PRRSV load (Zimmerman et al., 2019). While fetal lung tissue does contain PRRSV, the viral load is lower than in lymphatic tissue (Cheon and Chae, 2001). However, lung tissue was included in the pluck pool to potentially detect other pathogens within a single sample (Maes et al., 2023). Another possible cause for the reduced viral load could be the degradation of RNA due to the freeze-thaw process on the pluck-pool samples and the activity of ribonucleases. Nonetheless, it is noteworthy that all of the subsequently tested pluck-pools exhibited PRRSV-1-positive results. This finding implies that a certain degree of pooling and freeze/thawing can be deemed acceptable for diagnostic PCR purposes (Gerber et al., 2013). However, if whole-genome sequencing (WGS) is planned as a subsequent investigation, it should be noted that the success rate of WGS is substantially influenced by the viral load, particularly within tissues (Gagnon et al., 2021). Storing samples at -80°C and adding reagents with an RNA-preserving effect may be crucial, especially when WGS is intended.

When interpreting the PCV2-positive results, it is important to consider that PCV2 is a ubiquitous pathogen and is associated with

subclinical infection (PCV2-SI) as well as clinical conditions such as PCV2-reproductive disease (PCV2-RD) (Segalés and Sibila, 2022). qPCR results can serve as a viable proxy for diagnosing PCV2-RD (Segalés and Sibila, 2022). In the context of the present PCR methodology, a threshold of 10^9 PCV2 GE/g heart tissue is proposed to meet the PCV2-RD diagnostic criteria (Unterweger et al., 2021). However, the clinical impact of even lower PCV2 viral loads in compromising the piglet immune system is under scientific debate (Seo et al., 2014). The linear regression curve illustrated in Fig. 1B suggests a strong correlation between the viral load of the heart pools and those of the pluck-pools. It is noteworthy that the published threshold values relate to the examination of individual hearts, rather than the analysis of pooled samples (Segalés and Sibila, 2022; Unterweger et al., 2021).

In summary, the present study indicates that the use of pluck-pools from aborted fetuses can serve as valuable diagnostic material. A single extraction from multiple pooled organ samples analyzed by subsequent PCRs can increase efficiency and reduce workload in laboratories. Additional research is required to assess the feasibility of follow-up investigations (e.g. WGS) and to define the relevance of viral loads from a clinical perspective.

CRedit authorship contribution statement

Auer Angelika: Methodology, Validation, Writing – review & editing. **Dimmel Katharina:** Methodology, Validation, Writing – review & editing. **Rümenapf Till:** Methodology, Validation, Writing – review & editing. **Ladinig Andrea:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. **Kreutzmann Heinrich:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Schwarz Lukas:** Conceptualization, Data curation, Investigation, Writing – review & editing. **Unterweger Christine:** Conceptualization, Data curation, Investigation, Writing – review & editing.

Declaration of Competing Interest

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgments

Preliminary results were presented as an abstract at the 13th European Symposium of Porcine Health Management (ESPHM), Budapest, Hungary, 11–13 May 2022.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.tvjl.2024.106081](https://doi.org/10.1016/j.tvjl.2024.106081).

References

- Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schonheit, J., 2007. Association of myocarditis with high viral load of porcine circovirus type 2 in several tissues in cases of fetal death and high mortality in piglets. A case study. *Journal of Veterinary Diagnostic Investigation* 19, 368–375.
- Cheon, D.S., Chae, C., 2001. Distribution of porcine reproductive and respiratory syndrome virus in stillborn and liveborn piglets from experimentally infected sows. *Journal of Comparative Pathology* 124, 231–237.
- Christianson, W.T., 1992. Stillbirths, mummies, abortions, and early embryonic death. *Veterinary Clinics of North America: Food Animal Practice* 8, 623–639.
- Gagnon, C.A., Lalonde, C., Provost, C., 2021. Porcine reproductive and respiratory syndrome virus whole-genome sequencing efficacy with field clinical samples using a poly(A)-tail viral genome purification method. *Journal of Veterinary Diagnostic Investigation* 33, 216–226.
- Gerber, P.F., O'Neill, K., Owolodun, O., Wang, C., Harmon, K., Zhang, J., Halbur, P.G., Zhou, L., Meng, X.J., Opriessnig, T., 2013. Comparison of commercial real-time reverse transcription-PCR assays for reliable, early, and rapid detection of heterologous strains of porcine reproductive and respiratory syndrome virus in experimentally infected or noninfected boars by use of different sample types. *Journal of Clinical Microbiology* 51, 547–556.
- Hansen, M.S., Hjulsgaard, C.K., Bille-Hansen, V., Haugegaard, S., Dupont, K., Hogedal, P., Kunstmann, L., Larsen, L.E., 2010. Selection of method is crucial for the diagnosis of porcine circovirus type 2 associated reproductive failures. *Veterinary Microbiology* 144, 203–209.
- Hoffmann, B., 2016. AVID-Methodensammlung: VIR02-pCV-2-qPCR-FLI. (http://avid.dvg.net/fileadmin/Bilder/PDF_AVID_Alt/website/Methoden_ab_2016/AVID-Methode_VIR02_porcines_Circovirus_2_final.pdf) (Accessed 22 September, 2023).
- Kreutzmann, H., Stadler, J., Knecht, C., Sassu, E.L., Rucizka, U., Zablotzki, Y., Vatzia, E., Balka, G., Zaruba, M., Chen, H.W., Riedel, C., Rümenapf, T., Ladinig, A., 2022. Phenotypic characterization of a virulent PRRSV-1 isolate in a reproductive model with and without prior heterologous modified live PRRSV-1 vaccination. *Frontiers in Veterinary Science* 9, 820233.
- Maes, D., Peltoniemi, O., Malik, M., 2023. Abortion and fetal death in sows. *Reproduction in Domestic Animals* 58, 125–136.
- Pensaert, M.B., Sanchez Jr., R.E., Ladekjaer-Mikkelsen, A.S., Allan, G.M., Nauwynck, H. J., 2004. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Veterinary Microbiology* 98, 175–183.
- Reif, J., Renzhammer, R., Brunthaler, R., Weissenbacher-Lang, C., Auer, A., Kreutzmann, H., Fux, R., Ladinig, A., Unterweger, C., 2022. Reproductive failure in an Austrian piglet-producing farm due to porcine circovirus genotype 2d. *Acta Veterinaria Hungarica* 70, 149–155.
- Segalés, J., Sibila, M., 2022. Revisiting porcine circovirus disease diagnostic criteria in the current porcine circovirus 2 epidemiological context. *Veterinary Sciences* 9, 110.
- Seo, H.W., Park, C., Han, K., Chae, C., 2014. Effect of porcine circovirus type 2 (PCV2) vaccination on PCV2-viremic piglets after experimental PCV2 challenge. *Veterinary Research* 45, 13.
- Sinn, L.J., Klingler, E., Lamp, B., Brunthaler, R., Weissenböck, H., Rümenapf, T., Ladinig, A., 2016. Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria. *Porcine Health Management* 2, 28.
- Unterweger, C., Brunthaler, R., Auer, A., Fux, R., Weissenbacher-Lang, C., Ladinig, A., 2021. Reconsideration of the diagnostic criteria required for PCV2 reproductive disease. *The Veterinary Journal* 272, 105660.
- Unterweger, C., Kreutzmann, H., Bünger, M., Klingler, E., Auer, A., Rümenapf, T., Truyen, U., Ladinig, A., 2023. Litters of various-sized mummies (LVSM) and stillborns after porcine reproductive and respiratory syndrome virus type 1 infection - a case report. *Veterinary Sciences* 10, 494.
- Zimmerman, J.J., Dee, S.A., Holtkamp, D.J., Murtaugh, M.P., Stadejek, T., Stevenson, G. W., Torremorell, M., Yang, H., Zhang, J., 2019. Porcine reproductive and respiratory syndrome viruses (porcine arteriviruses). *Diseases of Swine*. Wiley-Blackwell, Hoboken, New Jersey, pp. 685–708.