

This is the peer-reviewed, manuscript version of an article published in *Veterinary Record*. The final version is available online via <http://dx.doi.org/10.1136/vr.104450>.

The full details of the published version of the article are as follows:

TITLE: Characterisation of porcine circovirus type 2 in porcine circovirus disease cases in England and Wales

AUTHORS: Sylvia S Grierson, Dirk Werling, Cornelia Bidewell and Susanna Williamson

JOURNAL TITLE: Veterinary Record

PUBLISHER: BMJ Publishing Group

PUBLICATION DATE: January 2018

DOI: 10.1136/vr.104450

VET RECORD: RESEARCH PAPER

Characterisation of porcine circovirus 2 in porcine circovirus disease cases in England and Wales

S.S. Grierson, D. Werling, C. Bidewell, S. Williamson

S Grierson, PhD,

Department of Virology, Animal and Plant Health Agency, Woodham Lane, Addlestone, Surrey, United Kingdom

D Werling BVetMed, DrMedVet, PhD, MRCVS

The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield Hertfordshire, United Kingdom

C Bidewell, BVetMed, MSc, MRCVS

S Williamson, BVetMed, PhD, MRCVS

Animal and Plant Health Agency, Rougham Hill, Bury St Edmunds, Suffolk, United Kingdom

Email for correspondence: Sylvia.Grierson@apha.gsi.gov.uk

ABSTRACT

Confirmed cases of porcine circovirus disease (PCVD) in GB have shown a steady decline since the availability of porcine circovirus 2 (PCV2) vaccines. However, PCVD is still sporadically diagnosed. We carried out a genotyping study to characterise PCV2 associated with confirmed PCVD cases in England and Wales from 2011 to January 2016 (n=65). A partial fragment of PCV2 genome encompassing open reading frame 2 (ORF2) was amplified and sequenced from 45 cases of PCVD. The majority of sequences were genotype PCV2b but four sequences were PCV2d. The significance of the emergence of PCV2d in England and elsewhere in the world is not yet known but may represent a global genotype shift.

INTRODUCTION

Disease associated with porcine circovirus 2 (PCV2) emerged in pigs in England from 1999 manifesting mainly as postweaning multisystemic wasting syndrome (PMWS). It rapidly became a significant cause of economic loss to the pig industry, due to both the effects of disease on pig productivity, health and welfare and the costs relating to interventions to treatment and control (Alarcon and others 2013). Following its initial emergence and spread, the clinical and pathological presentations of porcine circovirus diseases (PCVD) have become more diverse with enteric, hepatic, respiratory and even, nervous presentations described (Segales 2012). The pathogenesis of PCVD is still not fully understood; however the availability of commercial PCV2 vaccines in the UK since 2006 has effected a dramatic reduction on the impact of this viral infection (Chae 2012). Vaccination is widespread in commercial pig herds in Great Britain and has proven effective in improving health and productivity through control of clinical disease and subclinical effects (Kurmann and others 2011) (Young 2011).

PCV2 was first characterised in 1998 and subsequent analyses, including retrospective analyses showed that PCV2a predominated in the pig population initially but there was then a global genotype shift from PCV2a to PCV2b around 2003 (Beach and Meng 2012). Currently there are four recognised PCV2 genotypes, PCV2a, PCV2b, PCV2c and PCV2d. Interestingly, PCV2d was referred initially to as mutant PCV2b (Guo and others 2012) and has recently been reclassified as PCV2d (Xiao and others 2015). Based on these genotypic changes analysed, it appears that there may now be an ongoing global genotype shift from PCV2b to PCV2d (Xiao and others 2015). Furthermore, a fifth genotype, PCV2e has recently been proposed, based on the analysis of 10 novel sequences that were obtained from samples from 2006-2015 from the US and Mexico (Davies and others 2016). Until very recently PCV2c had only been detected in archived samples from Denmark (1980, 1987 and 1990) but there is now a report that it is circulating in feral pigs in Brazil (Franzo and others 2015a). There is no evidence that PCV2c is currently circulating in the commercial pig population.

There is no information available regarding the genotype of PCV2 currently circulating in the pig population in GB. Data obtained during the Royal Veterinary College (RVC) PMWS project (Alarcon and others 2011) had found that in 2008-2009, PCV2b was the main genotype circulating in pigs in England (D. Werling, personal communication).

The annual diagnostic rate of PCVD in GB has shown a steady decline. Occasional incidents are still diagnosed, which arise in either unvaccinated pigs or in herds where it was known, or strongly suspected, that groups of pigs had inadvertently not been vaccinated or had some other vaccine administration issue (APHA 2014). These incidents include some with unusual presentations, such as foetopathy (APHA 2015). Occasionally, incidents have been diagnosed where no reason was identified for the occurrence of PCVD in vaccinated pigs (Florins and others 2007). In order to investigate PCVD incidents, and obtain genotyping information for surveillance purposes, viruses associated with confirmed PCVD cases in England and Wales from 2011 to 2016 were characterised.

MATERIAL AND METHODS

The study investigated 65 diagnostic submissions submitted to the Animal and Plant Health Agency (APHA) Veterinary Investigation Centres from 2011 to January 2016 with confirmed diagnosis of PCVD. Diagnostic criteria for an individual pig were based on Sorden (2000) and Opriessnig and others (2007). They comprise of three components, all of which must be present; clinical signs, histological lesions (lymphoid depletion of tissue and/or histiocytic inflammation) and the presence of PCV2 antigen associated with the microscopic lesions.

For submissions from 2011-2013, one sample (one pig) per submission was investigated for this study while between 1 and 3 samples (from different pigs) were investigated for submissions from 2014-2016. Lymph node samples were used in preference to other tissues where available; otherwise, lung or heart samples were used. Samples were available either as sections cut from paraffin-embedded tissue blocks (PETB) or fresh tissues stored frozen at minus 20°C from most recent cases. When sections were cut from PETB, precautions were taken including use of new microtome blades for each sample to prevent cross contamination. Nucleic acid was extracted from PETB as described previously (Grierson and others 2004a). Digestion of the PETB sections was performed using Proteinase K (Ambion) in a digestion buffer composed of 100mM NaCl, 0.5% SDS, 10mM Tris-HCl (pH 8) and 1mM EDTA (pH 8). Nucleic acid was extracted from tissue using the QIAamp DNA Mini kit (Qiagen), and performed according to manufacturer's instructions. A 704bp fragment of the PCV2 genome, encompassing the entire coding sequence of open reading frame 2 (ORF2) was amplified using primers described in Fort and others (2007). The resulting amplicon was analysed by Sanger sequencing: phylogenetic analysis of ORF2 is representative of whole genome analysis (Olvera and others 2007). Sequences were assembled using SeqMan™ (DNASTAR) and subsequently

aligned using MegAlign™ (DNASTAR). Reference genotypes (PCV2a-PCV2d; Franzo and others 2015b) and proposed genotype PCV2e sequences (Davies and others 2016) were included in the sequence analysis. In addition, 30 PCV2b ORF2 sequences acquired during the RVC PMWS project (BB/FO18394/1) were included in the analysis (KY806000-KY806029). These sequences were obtained from samples collected in 2008-2009 from PMWS affected farms in England. Genetic distances were calculated using MEGA5 (Neighbor-Joining) (Tamura and others 2011).

RESULTS

ORF2 sequences were obtained from 45 of the 65 submissions tested (Table 1). In most cases, sequence obtained from more than one sample (pig) in a single submission were found to be identical, except for three instances for which two unique sequences were obtained (99.0-99.3% identity within a submission). A total of 48 sequences were obtained from 45 submissions (Table 1). All but four of the 48 ORF2 sequences were 702 nucleotides in length and encoding for a protein of 234 amino acids. Nucleotide sequence identity of these 44 sequences ranged from 92.6-100% (40 unique sequences; Genbank accession KY806070-KY806071). Phylogenetic analysis showed that sequences clustered with reference PCV2b strains and that there was no obvious clustering of sequences relative to those obtained from pigs in England in 2008-2009 (Figure 1).

Four sequences were 705 nucleotides in length as a result of a substitution in the stop codon and that encoded for an additional amino acid (Lysine). Nucleotide sequence identity of these four sequences ranged from 99.9-100% (2 unique sequences; Genbank accession KY806070-KY806071). These sequences clustered within genotype PCV2d (Figure 1).

Disease presentation for the majority of the 45 confirmed PCVD diagnoses comprised of typical PMWS clinical signs (wasting, with or without respiratory signs, with or without diarrhoea.). However, two submissions were of reproductive disease (one has been reported elsewhere (APHA 2015)). Both instances of reproductive disease were associated with PCV2b.

Information on farm PCV2 vaccination status was available for only 23 of the 45 submissions from which sequence data were obtained, and of these 52% of farms (12 of 23) did not vaccinate. Of the four submissions in which PCV2d was detected, three farms vaccinated (at 6-8 weeks of age), whereas the status of the fourth was unknown. The pigs in which PCV2d was detected were from farms in North Yorkshire and East Anglia.

DISCUSSION

It has been estimated that PCV2d originated around 20 years ago (Xiao and others 2015). However, it appears that its prevalence has increased in China (Cai and others 2012), and there are now increasing reports of its detection in North America, South America and Europe. Indeed, the virus was retrospectively detected in a sample from 1999 from Switzerland (Xiao and others 2015) and has been sporadically detected since then in countries such as Netherlands (2001-2002; (Grierson and others 2004b), China (2002) and Germany (Xiao and others 2015). The reports suggest there is a steady emergence of PCV2b and a slow genotypic shift from PCV2b to PCV2d (Xiao and others 2015). The sample set in this study is not yet sufficient to state that there is an increased trend of PCV2d in England and Wales, but continued monitoring of the PCV2 genotype from disease incidents may allow analysis in the future. PCV2d was detected in a sample from one pig farm from 2013 and three samples derived from two farms from 2014. The absence of detection in samples from 2015 and 2016 may simply be due to the small sample sizes (six and one submission diagnosed with PCVD respectively). The four PCV2d ORF2 sequences from 2013 and 2014 share high nucleotide identity and indeed three of the four were identical in ORF2 sequence. The PCV2d sequences were from two regions of England: the study analysed samples that had been submitted to eleven APHA Veterinary Investigation Centres.

The significance of the apparent emergence of PCV2d pigs in England is not yet known. There are currently conflicting evidence with regard to potential differences in virulence relative to PCV2a and PCV2b. Guo et al. (2012) reported enhanced virulence in weaned, 30-day old commercial piglets (more severe signs compatible with PMWS; more severe pathological lesions and higher viral loads; lower average daily weight gain) relative to challenge with PCV2a and PCV2b. Yet Opriessnig et al. (2014b) found no significant difference in virulence although they did not rule out there being some difference.

It has to be stressed that all licensed vaccines, despite being based on on the PCV2a genotype, show cross-protection against PCV2b (Fort and others 2008) and PCV2d (Opriessnig and others 2014a). Interestingly, PCV2d strains characterised from 1999 and 2001/2002 were from healthy pigs (Grierson and others 2004b; Xiao and others 2015) and indeed strain NL-control-4 was from a farm that had no history of PMWS or porcine dermatitis and nephropathy syndrome (PDNS) (Grierson and others 2004b). Reports of the detection of PCV2d have been associated with apparent vaccine breakdown and consequently the cross-protectiveness of the

PCV2a based vaccines was questioned (Opriessnig and others 2013b). However, as stated above, PCV2a-based vaccines have been shown to protect against PCV2d challenge (Opriessnig and others 2014a). To date there is no evidence that current vaccines are not effective against the circulating PCV2 genotypes in controlling disease, although they may be less effective in reducing the amount of virus (Opriessnig and others 2013a). It should be noted that instances of apparent vaccine failure may be due to issues related to the storage, handling, administration or timing of vaccination rather than a true lack of vaccinal efficacy. Also assessing the virulence of PCV2 strains in the field is not possible because of the variation in environment, management, concurrent pathogens and other factors influencing the severity of PCVD in pigs.

PCR products for sequencing were not obtained from 20 of the submissions tested although PCV2 was known to be present from histopathological and immunohistochemical investigations. This is not unexpected as a relatively large fragment of the PCV2 genome is amplified for characterisation (~740bp) and which reduces the sensitivity of detection of the PCR. The quality of the DNA extracted from the PETB will also have affected the ability to obtain PCR product and which will be affected by the sample quality prior to fixation and embedding amongst other factors (Grierson and others 2004a).

Although vaccination is effective in controlling disease it does not prevent virus infection establishing, therefore PCV2 infection will continue to circulate in the pig population albeit at a lower rate. APHA will continue to monitor for any change in clinical or epidemiological presentation of PCVD or vaccination failure in pigs in England and Wales.

ACKNOWLEDGEMENTS

This work was funded by Defra within the APHA project “Scanning surveillance for pig diseases in England and Wales (ED1200)”. The expertise of APHA veterinary and scientific colleagues in the Veterinary Investigation Centres and Lasswade is acknowledged as is the provision of diagnostic material to APHA by submitting private veterinary practitioners and their pig keeping clients. Archived samples from the RVC were a result of a grant ([BB/FO18394/1](#)) funded through the the BBSRC ‘Combating Endemic Diseases of Farmed Animals for Sustainability’ (CEDFAS) initiative, with contributions from BPEX, Biobest Laboratories and Zoetis Animal Health. We would like to thank Marie Walker (Lasswade) for organising the PETB sections for this study.

TABLE 1 Characterisation of ORF2 of PCV2 in cases of PCVD in England and Wales

Year	No. Submissions tested	No. Submissions from which sequence(s) obtained	PCV2b		PCV2d	
			No. Submissions	No. Sequences (unique ¹)	No. Submissions	No. Sequences (unique ¹)
2011	18	11	11	11 (8)	0	0
2012	14	9	9	9	0	0
2013	11	7	6	6	1	1
2014	15	13	10	11 ² (10)	3	3 (2)
2015	6	4	4	6 ³	0	0
2016	1	1	1	1	0	0
Total	65	45	41	44 (40)	4	4 (2)

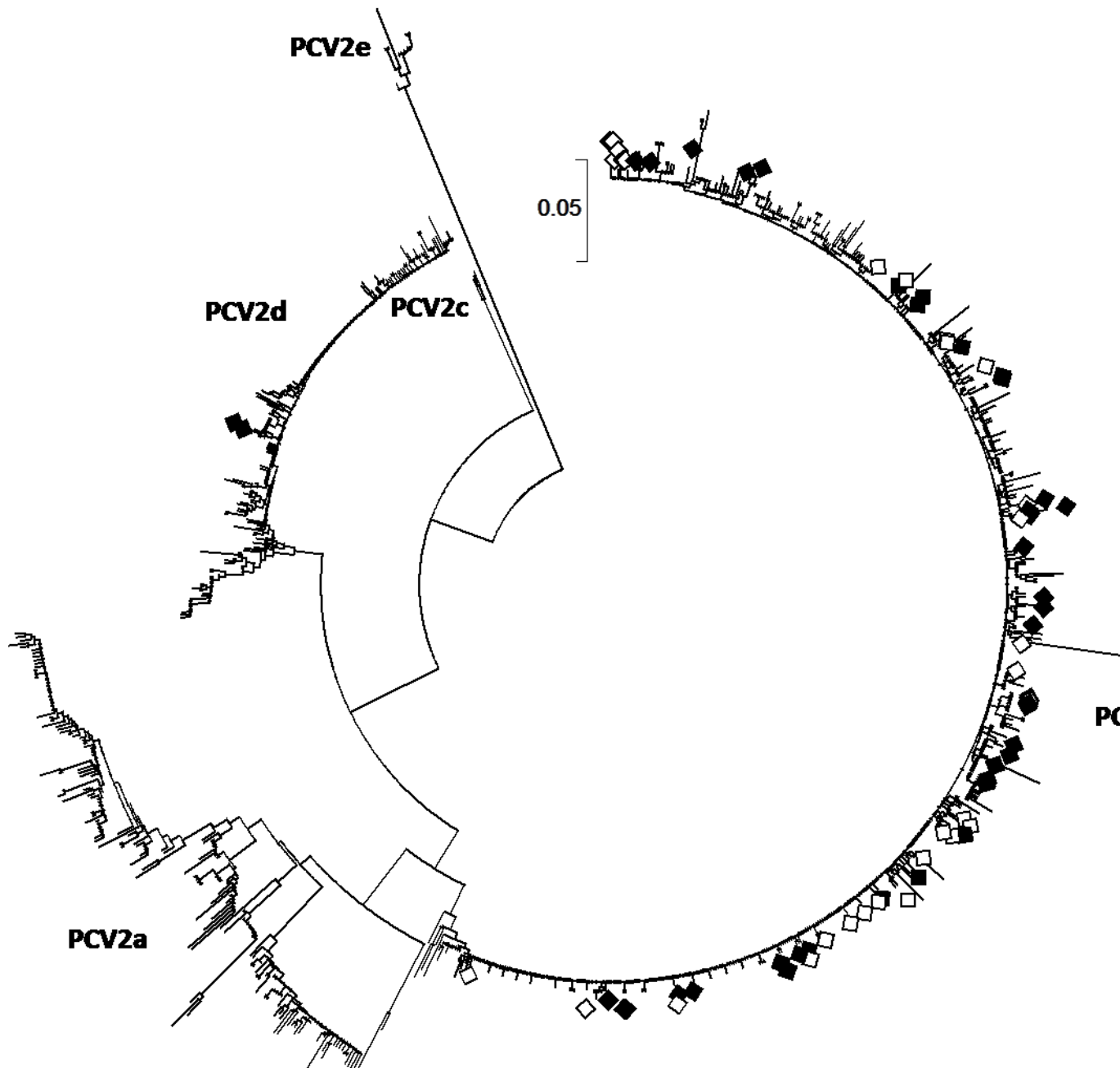
No., number

¹ Number of unique sequences per year if different to the No. Sequences

² Two unique sequences from one submission

³ Two unique sequences from each of two submissions

FIGURE 1 Phylogenetic dendrogram depicting genetic distance based on the complete coding sequence of ORF2 (702-708nt). Sequences were aligned using MegAlign (DNASTAR) and genetic distances calculated using MEGA5 (Neighbor-Joining). Analysis includes 902 reference genotypes (PCV2a-PCV2d) (Franzo and others 2015b) and 10 proposed genotype PCV2e sequences (Davies and others 2016). Study sequences are indicated (◆). Sequences from samples collected in England in 2008-2009 (RVC PMWS PROJECT) are indicated (◇).



REFERENCES

- ALARCON, P., RUSHTON, J. & WIELAND, B. (2013) Cost of post-weaning multi-systemic wasting syndrome and porcine circovirus type-2 subclinical infection in England - an economic disease model. *Prev Vet Med* 110, 88-102
- ALARCON, P., VELASOVA, M., WERLING, D., STARK, K. D., CHANG, Y. M., NEVEL, A., PFEIFFER, D. U. & WIELAND, B. (2011) Assessment and quantification of post-weaning multi-systemic wasting syndrome severity at farm level. *Prev Vet Med* 98, 19-28
- APHA (2014) Animal and Plant Health Agency. GB Emerging Threats Quarterly Report – Pig Diseases: January - March 2014, Q1, p.6.
https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/350666/pub-survrep-p0114.pdf. Accessed 01/12/2016 Access, 2014
- APHA (2015) Animal and Plant Health Agency. GB Emerging Threats Quarterly Report - Pig Diseases: January - March 2015, Q1, p.5.
https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/430707/pub-survrep-p0115.pdf. Accessed 01/12/2016 Access, 2015
- BEACH, N. M. & MENG, X. J. (2012) Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (PCV2). *Virus Res* 164, 33-42
- CAI, L., NI, J., XIA, Y., ZI, Z., NING, K., QIU, P., LI, X., WANG, B., LIU, Q., HU, D., YU, X., ZHOU, Z., ZHAI, X., HAN, X. & TIAN, K. (2012) Identification of an emerging recombinant cluster in porcine circovirus type 2. *Virus Res* 165, 95-102
- CHAE, C. (2012) Commercial porcine circovirus type 2 vaccines: efficacy and clinical application. *Vet J* 194, 151-157
- DAVIES, B., WANG, X., DVORAK, C. M., MARTHALER, D. & MURTAUGH, M. P. (2016) Diagnostic phylogenetics reveals a new Porcine circovirus 2 cluster. *Virus Res* 217, 32-37
- FLORINS, A., GILLET, N., ASQUITH, B., BOXUS, M., BURTEAU, C., TWIZERE, J. C., URBAIN, P., VANDERMEERS, F., DEBACQ, C., SANCHEZ-ALCARAZ, M. T., SCHWARTZ-CORNIL, I., KERKHOF, P., JEAN, G., THEWIS, A., HAY, J., MORTREUX, F., WATTEL, E., REICHERT, M., BURNY, A., KETTMANN, R., BANGHAM, C. & WILLEMS, L. (2007) Cell dynamics and immune response to BLV infection: a unifying model. *Front Biosci* 12, 1520-1531
- FORT, M., SIBILA, M., ALLEPUZ, A., MATEU, E., ROERINK, F. & SEGALES, J. (2008) Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. *Vaccine* 26, 1063-1071
- FRANZO, G., CORTEY, M., DE CASTRO, A. M., PIOVEZAN, U., SZABO, M. P., DRIGO, M., SEGALES, J. & RICHTZENHAIN, L. J. (2015a) Genetic characterisation of Porcine circovirus type 2 (PCV2) strains from feral pigs in the Brazilian Pantanal: An opportunity to reconstruct the history of PCV2 evolution. *Vet Microbiol* 178, 158-162
- FRANZO, G., CORTEY, M., OLVERA, A., NOVOSEL, D., CASTRO, A. M., BIAGINI, P., SEGALES, J. & DRIGO, M. (2015b) Revisiting the taxonomical classification of Porcine Circovirus type 2 (PCV2): still a real challenge. *Virology* 12, 131
- GRIERSON, S. S., KING, D. P., SANDVIK, T., HICKS, D., SPENCER, Y., DREW, T. W. & BANKS, M. (2004a) Detection and genetic typing of type 2 porcine circoviruses in archived pig tissues from the UK. *Arch Virol* 149, 1171-1183

GRIERSON, S. S., KING, D. P., WELLENBERG, G. J. & BANKS, M. (2004b) Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case-control study. *Res Vet Sci* 77, 265-268

GUO, L., FU, Y., WANG, Y., LU, Y., WEI, Y., TANG, Q., FAN, P., LIU, J., ZHANG, L., ZHANG, F., HUANG, L., LIU, D., LI, S., WU, H. & LIU, C. (2012) A porcine circovirus type 2 (PCV2) mutant with 234 amino acids in capsid protein showed more virulence in vivo, compared with classical PCV2a/b strain. *PLoS One* 7, e41463

KURMANN, J., SYDLER, T., BRUGNERA, E., BUERGI, E., HAESSIG, M., SUTER, M. & SIDLER, X. (2011) Vaccination of dams increases antibody titer and improves growth parameters in finisher pigs subclinically infected with porcine circovirus type 2. *Clin Vaccine Immunol* 18, 1644-1649

OLVERA, A., CORTEY, M. & SEGALÉS, J. (2007) Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. *Virology* 357, 175-185

OPRIESSNIG, T., GERBER, P. F., XIAO, C. T., HALBUR, P. G., MATZINGER, S. R. & MENG, X. J. (2014a) Commercial PCV2a-based vaccines are effective in protecting naturally PCV2b-infected finisher pigs against experimental challenge with a 2012 mutant PCV2. *Vaccine* 32, 4342-4348

OPRIESSNIG, T., MENG, X. J. & HALBUR, P. G. (2007) Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest* 19, 591-615

OPRIESSNIG, T., O'NEILL, K., GERBER, P. F., DE CASTRO, A. M., GIMENEZ-LIROLA, L. G., BEACH, N. M., ZHOU, L., MENG, X. J., WANG, C. & HALBUR, P. G. (2013a) A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection. *Vaccine* 31, 487-494

OPRIESSNIG, T., XIAO, C. T., GERBER, P. F. & HALBUR, P. G. (2013b) Emergence of a novel mutant PCV2b variant associated with clinical PCVAD in two vaccinated pig farms in the U.S. concurrently infected with PPV2. *Vet Microbiol* 163, 177-183

OPRIESSNIG, T., XIAO, C. T., GERBER, P. F., HALBUR, P. G., MATZINGER, S. R. & MENG, X. J. (2014b) Mutant USA strain of porcine circovirus type 2 (mPCV2) exhibits similar virulence to the classical PCV2a and PCV2b strains in caesarean-derived, colostrum-deprived pigs. *J Gen Virol* 95, 2495-2503

SEGALÉS, J. (2012) Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. *Virus Res* 164, 10-19

SORDEN, S. D. (2000) Update on porcine circovirus and porcine multisystemic wasting syndrome (PMWS). *J Swine Health Prod* 8, 133-136

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28, 2731-2739

XIAO, C. T., HALBUR, P. G. & OPRIESSNIG, T. (2015) Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. *J Gen Virol* 96, 1830-1841

YOUNG, M. G., CUNNINGHAM, G.L., SANFORD, S.E., (2011) Circovirus vaccination in pigs with subclinical porcine circovirus type 2 infection complicated by ileitis. *J. Swine Health Prod.* 19, 175-180

