Expression of PCV2 antigen in the ovarian tissues of gilts

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ABSTRACT. The present study was performed to determine the expression of porcine circovirus type 2 (PCV2) antigen in the ovarian tissue of naturally infected gilts. Ovarian tissues were obtained from 11 culled gilts. The ovarian tissues sections were divided into two groups according to PCV2 DNA detection using PCR. PCV2 antigen was assessed in the paraffin embedded ovarian tissue sections by immunohistochemistry. A total of 2,131 ovarian follicles (i.e., 1,437 primordial, 133 primary, 353 secondary and 208 antral follicles), 66 atretic follicles and 131 corpora lutea were evaluated. It was found that PCV2 antigen was detected in 280 ovarian follicles (i.e., 239 primordial follicles, 12 primary follicles, 10 secondary follicles and 19 antral follicles), 1 atretic follicles and 3 corpora lutea ($P<0.05$). PCV2 antigen was detected in primordial follicles more often than in secondary follicles, atretic follicles and corpora lutea ($P<0.05$). The detection of PCV2 antigen was found mainly in oocytes. PCV2 antigen was found in both PCV2 DNA positive and negative ovarian tissues. It can be concluded that PCV2 antigen is expressed in all types of the ovarian follicles and corpora lutea. Further studies should be carried out to determine the influence of PCV2 on porcine ovarian function and oocyte quality.
KEY WORDS: follicle, gilt, immunohistochemistry, ovary, porcine circovirus type 2 (PCV2)

Porcine circovirus type 2 (PCV2) is a small circular single stranded DNA virus. In recent years, PCV2 has been associated with many disease manifestations in all ages of pigs, so-called PCV2-associated disease (PCVAD) [8]. PCV2 is a primary cause of post-weaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome and PCV2-associated reproductive failure [2, 9, 14]. PCV2-associated disease has a significant economic impact on the swine industry worldwide. Furthermore, PCV2 also interacts with other pathogens, e.g., porcine parvovirus and porcine reproductive and respiratory syndrome virus, causing several complicated clinical symptoms in pigs [8]. Under field conditions, PCV2 serological detection for assessing vaccination compliance is being intensively investigated to control and minimize the clinical signs caused by PCVAD [14]. Although studies on the influence of PCV2 as well as vaccination efficacy are well-established in nursery and fattening pigs, additional knowledge concerning PCV2-associated reproductive failure is still required. In practice, three PCV2 vaccination regimens have been applied, i.e., sow and/or piglet vaccination [7]. However, the efficacy of the three vaccination regimens is still controversial [7]. In some cases, vaccinating against PCV2 in gilts and sows failed to improve reproductive performance, e.g., the farrowing rate and litter size [3]. In North America, PCV2 vaccination is still highly recommended in replacement gilts at least 2–3 weeks before entering the breeding facility [3]. On the other hand, studies have clearly confirmed that PCV2 infection causes reproductive failure in pregnant gilts and sows [3, 12, 13]. To our knowledge, the mechanism of PCV2 infection on the reproductive apparatus in gilts and sows is still not fully understood. Madson and Opriessnig [3] found that PCV2 infection during early, mid or late stage gestation in sows causes one or many characteristics of reproductive failure, e.g., early embryonic death, pseudopregnancy, irregular return to estrus, small litter size, mummified fetuses, abortion, stillborn piglets, weak born piglets and/or delayed farrowing. However, infected sows may also farrow a normal litter [3]. A previous study demonstrated that the embryonic survival rate of PCV2-exposed embryos is significantly lower than that of negative control embryos (6.4% versus 65.4%) [5]. Furthermore, PCV2 DNA has also been detected in 11% of cumulus-oocyte complexes and in 22% of follicular fluid samples from PCV2 antibody-positive sows by nested polymerase chain reaction assays [1]. Recently, PCV2 infected oocytes have been found to increase the risk of producing PCV2 contaminated embryos using the somatic cell nuclear transfer system [17]. The present study was performed to determine the expression of PCV2 antigen in different compartments of the ovary in PCV2-infected gilts.

Ovarian tissues were obtained from 11 culled gilts from our previous study [15]. The paraffin embedded ovarian tissues were cut into 4 μm thick sections and were divided into two groups according to previously identified PCV2 DNA detection, i.e. the PCV2-infected gilts (n=6) and non-infected gilts (n=5) [9]. Historical data and blood samples were collected from the gilts before culling. The serum samples were tested for PCV2 antibody titers using a commercial competitive enzyme-linked immunosorbent assay test kit

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(Synbiotics, Lyon, France). The PCV2 titers were classified as negative (<550) or positive (≥550) [9].

Immunohistochemistry was applied to all tissue sections to determine the expression of PCV2 antigen. The immunohistochemistry protocol was carried out according to our previous study [9]. Briefly, 4 μm-thick sections of paraffin-embedded tissue were placed on 3-aminopropyl-triethoxysilane-coated slides. The sections were deparaffinized with xylene and rehydrated by a graded ethanol series. Antigen retrieval was carried out by heating three times in a microwave oven at 250 W for 5 min in 0.01 M citrate buffer, pH 6.0. Blocking of the peroxidase reaction was performed by incubating the tissue samples with freshly prepared 5 ml of 30% H_2O_2 in 45 ml of methanol at room temperature for 20 min. Non-specific antigenic sites were blocked in 1.0% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 30 min at room temperature. The sections were incubated with rabbit polyclonal anti-PCV2 primary antibody GTX128120 (GeneTex, Inc., Irvine, CA, U.S.A.) at a dilution of 1:200 and incubated at 4°C for 24 hr. After that, the sections were washed three times in PBS and incubated with biotinylated goat anti-rabbit secondary antibodies (Vector Laboratories) at a dilution of 1:200 for 30 min. The avidin-biotin-peroxidase complex method was used to identify PCV2 antigens in the tissue samples. A solution of diaminobenzidine (Vector Laboratories) was used to develop the visualization of PCV2. The sections were counterstained with hematoxylin and mounted. The tissues were evaluated under a light microscope at magnifications of 100 × to 400 ×. The PCV2 antigens were localized by the brown intranuclear staining in the tissue sections. The intestine of a PCV2-infected pig served as the positive control. For the negative control, the primary antibody was omitted.

In all tissue sections, a total of 2,131 ovarian follicles (i.e., 1,437 primordial follicles, 133 primary follicles, 353 secondary follicles and 208 antral follicles), 66 atretic follicles and 131 corpora lutea were evaluated. Immunohistochemical staining of PCV2 antigen was determined by searching for brown staining in the nucleus or cytoplasm of all available follicles, corpora lutea and other tissue compartment of the ovarian tissues (Fig. 1). The follicles/corpora lutea were interpreted as positive, if they contained at least one positive cell (brown staining, Fig. 1).

The statistical analysis was carried out by using the Statistical Analysis System software (SAS version 9.0, SAS Institute, Cary, NC, U.S.A.). Continuous data are presented as mean ± SD, and categorical data are presented as percentage. The percentage of PCV2 expression in different compartments of the ovarian tissues (i.e., follicles, atretic follicles and corpora lutea) was compared within the ovary by using paired t tests. The difference in PCV2 antigen expression between PCV2-infected and non-infected ovarian tissues was compared by unpaired t tests. $P<0.05$ was considered statistically significant.

The summary of reproductive data of the gilts included in the experiment is presented in Table 1. On average, the gilts exhibited first standing estrus at 220 days of age and entered the breeding house at 232 days of age. The age and body weight at slaughter were 278 days and 152 kg. Of all the gilts (n=11), PCV2 antibody titers was positive in seven gilts, and PCV2-DNA was detected in six gilts. PCV2 immunohistochemical staining was detected in the ovarian tissues of all the gilts.

In the ovarian tissues, the immunohistochemical staining of PCV2 antigen was found mainly in oocytes (Fig. 1). The PCV2 antigen was detected in 13.5% of normal follicles (n=280), 1.8% of atretic follicles (n=1) and 3.8% of corpora lutea (n=3) ($P<0.05$). Of all the follicles, PCV2 antigen was found in 22.2% of primordial follicles, 10.2% of primary follicles, 3.2% of secondary follicles and 8.8% of antral follicles (Table 2). PCV2 antigen was detected in primordial follicles more than secondary follicles, corpora lutea and atretic follicles ($P<0.05$). PCV2 antigen was detected in both PCV2-DNA positive and negative ovarian tissues. No significant differences in PCV2 antigen detection were found in any follicle types and corpora lutea between PCV2 DNA positive and DNA negative ovarian tissues (Table 3).

The present study is the first report to clearly demonstrate the expression of PCV2 antigen in different stages of follicles (i.e., from primordial to antral follicles) of gilts. This indicates that the oocyte is one of the target cells of PCV2 infection. In our previous study, PCV2 DNA was detected in 30% (21/70 gilts) of ovarian tissue samples and in 45.1% (46/102 gilts) of uterine tissue samples in naturally PCV2 infected replacement gilts [9]. Nevertheless, no pathological lesion in relation to PCV2 DNA detection has been observed in either ovarian or uterine tissues [2, 9]. The present study shows that PCV2 antigen is expressed mainly in the oocytes. A previous study demonstrated that PCV2 is able to enter mature oocytes via a damaged zona pellucida and is able to reduce the developing competency of oocytes [17]. Furthermore, embryos with an intact zona pellucida are resistant to PCV2 infection, but zona pellucida-free porcine morulae and blastocysts are susceptible to the virus; susceptibility increases during embryonic development [6]. Previous studies have clearly demonstrated the impact of PCV2 infection on mature porcine oocytes and early embryonic stages. However, the impact of PCV2 infection on the early stages of oocytes (as observed in the present study) and the qualities of ovulated oocytes has not been determined. In practice, vaccination of young gilts before PCV2 exposure might help to reduce the severity of PCV2-infected reproductive apparatus before first insemination [3]. Furthermore, this may help to reduce the proportion of replacement gilts carrying PCV2 to pregnant sows when they are sent to the breeding house. The present findings indicate that awareness of PCV2 infection during the early stages of follicular development in replacement gilts should be addressed.

In the present study, PCV2 antigen was mainly expressed in oocytes within primordial follicles more than other types of follicles. The reason might be that the majority of follicles in pubertal gilt ovarian tissue are primordial follicles (64.2%), while 32.7% are primary and secondary follicles and 3.1% are antral follicles [11]. Furthermore, the pre-granulosa cells surrounding oocytes in primordial follicles are thinner than in more advanced follicle stages [11]. Therefore, penetration
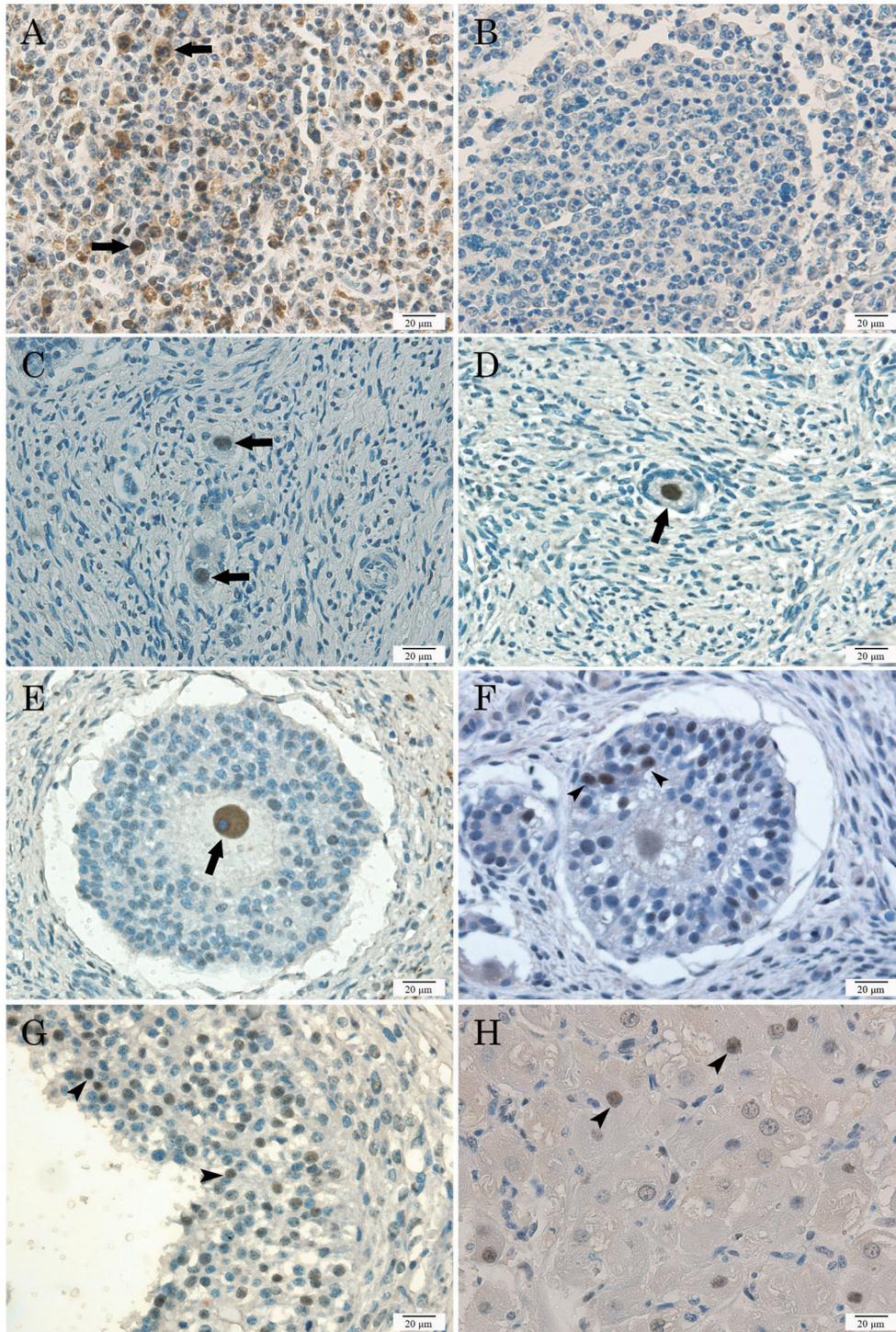
Fig. 1. Immunohistochemical staining of porcine circovirus type 2 (PCV2) antigen in the ovarian tissues of gilts. (A) positive control, PCV2 positive lymph node; (B) negative control; (C) positive PCV2 immunostaining of oocyte in primordial follicle; (D) positive PCV2 immunostaining of oocyte in primary follicle; (E) positive PCV2 immunostaining of oocyte in secondary follicle; (F) positive PCV2 immunostaining of granulosa cells in secondary follicle; (G) positive PCV2 immunostaining of granulosa cells in antral follicle; (H) positive PCV2 immunostaining in nucleus of luteal cells.

Table 1. Descriptive statistics on the reproductive data of culled gilts (n=11)

Variables	Mean ± SD	Range
Age at first observed estrus (days)	220 ± 15	190–243
Age at entry into the herd (days)	232 ± 13	204–256
Age at culling (days)	278 ± 26	246–332
Interval from entry to culling (days)	49 ± 20	22–95
Average daily gain (g/day)	542 ± 43	486–626
Number of ovulations	17.2 ± 3.6	13–24
Weight of ovary (g)	6.6 ± 3.0	3.0–12.0

Table 2. Porcine circovirus type 2 (PCV2) antigen expression in porcine ovarian tissue

Part of ovarian tissue	Number of observations	Number of positive observations	Percentage
Primordial follicles	1,437	239	22.2[a]
Primary follicles	133	12	10.2[a,b]
Secondary follicles	353	10	3.2[b]
Antral follicles	208	19	8.8[a,b]
Corpora lutea	131	3	3.8[b]
Atretic follicles	66	1	1.8[b]

a, b) Different superscripts within columns indicate significant differences ($P<0.05$).

Table 3. Percentage of porcine circovirus type 2 (PCV2) antigen expression in ovarian tissue with and without PCV2 DNA detection

Part of ovarian tissue	PCV2 antigen expression (%)		P value
	PCV2 DNA negative	PCV2 DNA positive	
Primordial follicles	24.0	20.8	ns
Primary follicles	21.5	0.8	0.059
Secondary follicles	2.1	4.1	ns
Antral follicles	14.8	3.9	ns
Corpora lutea	3.3	4.2	ns
Atretic follicles	0	3.3	ns
All	16.0	10.2	ns

* ns: Not significant ($P>0.1$).

of the PCV2 virus through the cells surrounding primordial follicles, leading to oocyte infection, might be easier than at advanced follicle stages. Nevertheless, less PCV2 antigen expression was found in the cells surrounding oocytes compared to oocytes (Fig. 1). PCV2 immunostaining in porcine ovarian tissues is rather different from the immunostaining of porcine reproductive and respiratory syndrome virus (PRRSV) in the ovarian tissues [16], obtained from the same group of gilts used in the present study. The PRRSV antigen is detected mainly in macrophages within the ovarian tissues and in granulosa cells rather than oocytes [10]. These findings may indicate differences in the pathogenesis of PCV2 and PRRSV infection in porcine ovaries. As can be seen from the staining pattern, PCV2 might affect the quality and developing competency of oocytes. In pregnant sows, PCV2 infection also has a direct effect on pathological changes in fetuses, e.g., infarction of the heart muscle, and can cause fetal death [4]. A recent study [17] found that PCV2-infected embryos and viral-positive cells were detected after the PCV2 virus infected zona pellucida-damaged oocytes. Furthermore, the blastocyst rate of PCV2 infected oocytes with a damaged zona pellucida was lower than that with an intact zona pellucida [17]. In addition, at the blastocyst stage, zona pellucida injury also leads to an increased apoptotic index of PCV2-infected embryos [17]. These data indicate that PCV2 potentially causes reproductive dysfunction in the porcine ovary via many mechanisms.

It can be concluded that the PCV2 antigen expressed in all types of ovarian follicles and corpora lutea. Immunohistochemistry is an effective tool for the detection of PCV2 an-

tigen in the porcine ovary. Further studies should be carried out to determine the influence of PCV2 on porcine ovarian functions as well as oocyte quality.

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