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Limited susceptibility of three different mouse (*Mus musculus*) lines to Porcine circovirus-2 infection and associated lesions

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Abstract

Porcine circovirus associated disease (PCVAD), a major global problem for pork producers, is characterized microscopically by depletion and histiocytic replacement of follicles in the lymphoid tissues. The objectives of this study were to determine 1) if Porcine circovirus-2 (PCV-2) inoculated mice *(Mus musculus)* can develop PCV-2 associated lymphoid lesions and serve as a model for PCVAD, and 2) if differences in PCV-2 host susceptibility exist among mice lines. Three groups ($n = 48$/group) of 4-wk-old male mice were used: BALB/c, C57BL/6, and C3H/HeJ. A 2×2 factorial analysis was designed for each group using PCV-2 inoculation and keyhole limpet hemocyanin in incomplete Freund's adjuvant injections on day 0 and 7 as factors. Necropsies were performed on days 12, 17, 22, 27, 32, and 37. Serum samples collected at each necropsy tested negative for anti-IgG PCV-2 antibodies in all mice at all time points by 2 different PCV-2 enzyme-linked immunosorbent assays (ELISA). The PCV-2 DNA was detected by polymerase chain reaction (PCR) in 93% (100/108) of tissues and 42.6% (46/108) of serum samples from PCV-2-inoculated mice from days 12 to 37. Microscopic lesions consistent with PCV-2 infection were not observed in any mice and PCV-2 DNA and PCV-2 antigen were not detected in tissues by in-situ-hybridization or immunohistochemistry assays, respectively. Based on incidence of PCV-2 DNA in serum samples, the C57BL/6 mouse line was more resistant to PCV-2 infection than the other lines. The results indicate the mouse model likely has limited utility to advance understanding of the pathogenesis of PCV-2 associated lesions, but mice could potentially be important in the epidemiology of PCV-2.

Résumé

La maladie associée au circovirus porcin (PCVAD) est un problème majeur global pour les producteurs de porcs et est caractérisée en microscopie par une déplétion des follicules lymphoïdes et une prolifération histiocytaire dans les tissus lymphoïdes. Les objectifs de la présente étude était de déterminer (1) si des souris (Mus musculus) *inoculées avec le circovirus porcin de type-2 (PCV-2) développent des lésions lymphoïdes associées au PCV-2 et pourraient servir de modèle pour PCVAD, et (2) si des différences de susceptibilité au PCV-2 existent entre les lignées de souris. Trois groupes (n = 48/groupe) de souris mâles ont été utilisés : BALB/c, C57/BL/6 et C3H/HeJ. Pour chaque groupe, le design expérimental consistait en une analyse factorielle 2×2 utilisant comme facteurs l'inoculation de PCV-2 et d'hémocyanine de patelle dans de l'adjuvant incomplet de Freund aux jours 0 et 7. Des nécropsies ont été effectuées aux jours 12, 17, 22, 27, 32, et 37. Des échantillons de sérum ont été prélevés à chaque nécropsie et se sont avérés négatifs, par deux méthodes immuno-enzymatiques différentes, pour la présence d'anticorps anti-IgG PCV-2 chez toutes les souris à tous les moments d'échantillonnage. De l'ADN de PCV-2 a été détecté par PCR dans 93 % (100/108) des tissus et 42,6 % (46/108) des échantillons de sérum provenant des souris inoculées avec PCV-2 et prélevés entre les jours 12 et 37. Aucune lésion microscopique compatible avec celles observées lors d'infections par PCV-2 n'a pas été vue chez aucune des souris, et l'ADN ou l'antigène PCV-2 n'ont pas été détectés dans les tissus, respectivement, par hybridation* in situ *ou analyses immuno-histochimiques. Basé sur l'incidence de l'ADN de PCV-2 dans les échantillons de sérum, la lignée de souris C57/BL/6 était plus résistante à l'infection par PCV-2 que les autres lignées. Les résultats indiquent que le modèle murin a une utilité limitée pour aider à la compréhension du la pathogénèse des lésions associées au PCV-2, mais que les souris pourraient potentiellement avoir une importance dans l'épidémiologie de PCV-2.*

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Introduction

Porcine circovirus (PCV) is a small, circular single-stranded DNA virus of the family *Circoviridae* (1). Two genera, *Circovirus* and *Gyrovirus* comprise this family. The genus *Gyrovirus* has one member, Chicken anemia virus, while the genus *Circovirus* includes Porcine circovirus-1 (PCV-1), Porcine circovirus-2 (PCV-2), pigeon circovirus, canary circovirus, goose circovirus, duck circovirus, and beak and feather disease virus (1). To date, pigs are the only mammalian host known to be infected by a member of the *Circoviridae* family. Several studies have reported that mice can become infected and/or harbor the virus but no host specific viruses have been identified for mammals other than swine (2–4).

Drawbacks in PCV-2 research include difficulty in finding source herds of PCV-2 antibody and virus-free pigs, the low incidence of clinical disease and PCV-2 associated lesions when commercially available pigs are experimentally infected with PCV-2, and the high cost of trials with large numbers of pigs (5). There is also evidence of host-related variation in susceptibility to PCV-2 even within the same breed and line of pigs (6). Conversely, mice that are PCV-2 antibody and virus free are readily available, the major mouse lines used frequently today (BALB/c, C57BL/6, and C3H/HeJ) are inbred strains (less concern of host-related variation), and more animals can be included in the trial at minimal expense compared with pigs.

Three studies have investigated the susceptibility of mice for PCV-2 infection. One study using BALB/c mice found detectable PCV-2 nucleic acids in the nuclei and cytoplasm of histiocytes and apoptotic cells in germinal centers of lymph follicles as well as in hepatocytes in the liver (2). This group also demonstrated mild lymphoid depletion and histiocytic infiltration of lymphoid follicles. The mice in this study were infected intraperitoneally as well as intranasally with a PCV-2 isolate that had been passaged 4 times in cell culture and originated from a case with naturally occurring PCVAD. A 2nd study by the same group demonstrated that BALB/c mice became infected with PCV-2 resulting in apoptosis in the spleen, lymph node, and Peyer's patches. The authors concluded that while mice became infected with PCV-2, the pathogenesis and associated lesions were not the same as in the pig model (3). Another study inoculated Crl:CD1 (ICR) mice intraperitoneally with the 4th cell culture passage of a PCV-2-isolate from a case of naturally occurring PCVAD (4). Despite the detection of PCV-2 DNA in sera, there was no evidence for association of PCV-2 with microscopic lesions; however, relevant lymphoid tissues (tonsil, lymph node, spleen) that are the major sites of PCV-2 associated lesions in pigs were not examined.

In the aforementioned studies, mice were inoculated with PCV-2 isolates that were not well characterized; therefore, it is unknown if these isolates are able to induce the PCV-2 characteristic lesions in pigs. The 1st objective of the current study was to determine if mice inoculated with a well-characterized, homogenous virus stock derived from an infectious PCV-2 clone can induce the hallmark lymphoid lesions of PCVAD as we have previously demonstrated in the pig model (7–10). The 2nd objective was to determine if there are differences in susceptibility to PCV-2 infection among different lines of mice.

Materials and methods

Mice and housing

One-hundred-forty-four, 4-week-old, male, specific pathogen free mice (BALB/c *n* = 48, C57BL/6 *n* = 48, C3H/HeN *n* = 48) were obtained from a commercial source (Harlan Sprague Dawley, Indianapolis, Indiana, USA). This source routinely performs serological assays for multiple agents (a full list of the 22 assays can be found at http//:www.harlan.com), bacterial workups for *Helicobacter* spp. as well as for respiratory and enteric organisms, and parasitic assays for endo-, ecto-, and enteric parasites. Upon arrival, mice were brought to the research facility at Iowa State University and randomly assigned to 30 cages containing 3–5 mice in each cage. The cages were standard microisolators and contained ground corn cob bedding, a self feeder, and an automatic watering device. Bedding, water, and feed were routinely autoclaved and the water was acidified. The diet used was a standard mouse diet (Teklad 7004; Harlan Teklad, Madison, Wisconsin, USA). The temperature in the room was kept at 22°C and a 12-hour light cycle was implemented. Animal care and use protocols for this study were approved by the Iowa State University Institutional Animal Care and Use Committee.

Experimental design

On arrival, the 144 mice were inspected, randomly placed into cages, and allowed to acclimate for 7 d. The cages were randomly assigned to treatment groups by using a computer randomization program. Each of these groups of 48 mice were analyzed in a 2×2 factorial study design using PCV-2 inoculation and administration of keyhole limpet hemocyanin in incomplete Freund's adjuvant (KLH/ICFA) as design factors. A summary of the trial design, including the number of mice used in each treatment group, is presented in Table I. On day 0, all mice were weighed and, for identification purposes, ear clipped. Additionally, a percentage of mice were injected with KLH/ICFA (24 of 48 per mouse line) and/or inoculated with PCV-2 (36/48 per mouse line; outlined in Table I). On day 7, the same mice were re-injected with KLH/ICFA and/or inoculated with a 2nd dose of PCV-2. On days 12, 17, 22, 27, 32, and 37 necropsies were performed on 8 mice from each group (Table I). At the time of necropsy, serum samples were collected for PCV-2 enzyme-linked immunosorbent assay (ELISA) and PCV-2 polymerase chain reaction (PCR). Additionally, tissue samples were collected for histopathology, PCV-2 in-situ hybridization (ISH), and immunhistochemistry (IHC). The mice were monitored daily and evaluated for signs of respiratory disease or lethargy throughout the trial.

PCV-2 inoculation

A homogenous infectious stock of PCV-2 isolate ISU-40895 (11) was generated via direct transfection of PK-15 cells with an infectious clone of PCV-2 as previously described by Fenaux et al (7). The infectivity titer was determined by inoculation of PK-15 cells with serially diluted virus stock, followed by an immunofluorescence assay (IFA) with PCV-2 specific antibodies as described (7). The virus inoculum contained infectious PCV-2 at a final titer of $0.5 \times 10^{4.25}$ 50% tissue culture infective dose ($TCID_{50}$) per mL. The inoculum was tested negative for the presence of porcine parvovirus and PCV-1 nucleic

Table I. Forty-eight mice from each mouse line (BALB/c, C57BL/6 and C3H/HeJ) were analyzed in the following 2 × 2 factorial study design using PCV-2 inoculation and keyhole limpet hemocyanin in incomplete Freund's adjuvant (KLH/ICFA) as design factors. For each line, the total number (n) of animals per group and the numbers of mice euthanized at each day post-inoculation are provided

| Inoculation[a] | KLH/ICFA[b] | n | Number of mice necropsied on each day post-inoculation |
			12	17	22	27	32	37
—	—	6	1	1	1	1	1	1
—	Yes	6	1	1	1	1	1	1
PCV-2	—	18	3	3	3	3	3	3
PCV-2	Yes	18	3	3	3	3	3	3
Total number of mice		48	8	8	8	8	8	8

[a] Inoculation with PCV-2 was done on days 0 and 7.
[b] KLH/ICFA administration was done on days 0 and 7.

acids by PCR (11,12). Mice were inoculated with 0.05 mL of the inoculum intramuscularly. Two intramuscular injections were given on each of days 0 and 7, one in the prescapular region and one into the epaxial musculature. At each time point, mice were additionally dosed with 0.02 mL intranasally. These routes have been shown to be successful in pigs. Control mice were similarly sham-inoculated with 0.12 mL of sterile saline buffer.

KLH/ICFA administration

In pigs, it has been demonstrated that immune stimulation with KLH/ICFA induces increased replication of PCV-2 and increased severity of PCV-2 associated lesions (13). In order to assess the effect of immune stimulation on the severity of PCV-2 infection in mice, administration of KLH/ICFA was included as a design factor: keyhole limpet hemocyanin (A.G. Scientific, San Diego, California, USA), a foreign antigen, was emulsified in incomplete Freund's adjuvant (Sigma-Aldrich, Saint Louis, Missouri, USA). Selected mice (Table I) were injected with 0.05 mL KLH/IFA subcutaneously into the tail base and with 0.05 mL KLH/IFA subcutaneously into the posterior cervical area; the total dose was 50 mg of KLH (14).

Serology

Blood samples were collected at necropsy. The serum samples were tested by an indirect IgG PCV-2-ELISA based on the recombinant open reading frame 2 capsid protein of PCV-2 (15). Samples were considered to be positive if the calculated sample-to-positive ratio was ≥ 0.2. In addition, a commercially available competitive IgG PCV-2 ELISA (SERELISA PCV2 AB Mono blocking ELISA IgG; Synbiotics, Lyon, France) was used for confirmation of test results on all samples.

DNA extraction and PCR

The DNA-extraction on serum samples collected at each necropsy day was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). The DNA extracts were used for PCV-2 PCR yielding a 547 base pairs (bp) product as previously described

(12). In addition, the amount of PCV-2 DNA in serum samples from all mice was determined by quantitative real-time PCR (10).

Necropsy

Necropsies were performed on randomly selected mice on days 12, 17, 22, 27, 32, and 37 (Table I). Gross abnormalities such as lymph node enlargement were recorded. Sections of lymph nodes (superficial inguinal, popliteal, axillary, cervical, and mesenteric), thymus, ileum, kidney, colon, heart, pancreas, spleen, liver, brain, lung, femur, and sternum were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

Histopathology

Microscopic lesions were evaluated in a blinded fashion by a veterinary pathologist. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 to 3 (0 = normal; 3 = severe). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 to 3 (0 = none to 3 = severe).

PCV-2 ISH and IHC

The ISH for detection of PCV-2 specific DNA was performed on all formalin-fixed and paraffin-embedded tissue sections by using a single-stranded 40-base oligonucleotide probe essentially as previously described (16). Similarly, IHC was done on all formalin-fixed and paraffin-embedded tissues sections using rabbit polyclonal anti-PCV-2 serum as described (17).

Statistical analysis

Summary statistics were calculated for all mice within each line to assess the overall quality of the data. Analysis of variance (ANOVA) was used for cross-sectional assessment of treatment factors. The significance level was $P < 0.05$ followed by pairwise testing using the Tukey-Kramer adjustment to identify the groups that were different. Pearson's chi-squared analysis was used on histopathologic data, serum PCR, and tissue PCR data to test if the lesion rate was similar between groups.

Results

Clinical disease

None of the mice were clinically affected during the study. Specifically, no evidence of weight loss, wasting, or respiratory disease was noted in any of the mice.

Presence of PCV-2 DNA

All serum samples obtained from the non-inoculated control mice were negative for PCV-2 specific nucleic acids as analyzed by gel-based and quantitative real-time PCR. In the PCV-2 inoculated mice, PCV-2 DNA was detected first on day 12 in pooled tissue samples and in serum samples. Pooled tissue samples remained positive through day 37 in all groups (Table II). Serum samples were also positive through day 37 for the BALB/c mice treatment groups (Table III). The C57BL/6 mice had significantly fewer ($P = 0.008$)

Table II. Incidence of PCV-2 positive tissue pool (liver, lymph nodes, lung, spleen) numbers from PCV-2 inoculated mice detected by gel-based PCV-2 PCR analysis (number of PCV-2 PCR positive tissue pools/total number tissue pools analyzed)

Line		Day post-inoculation						
		12	17	22	27	32	37	Total
BALB/c	KLH/ICFA[a]	3/3	2/3	3/3	3/3	3/3	3/3	17/18
	—	3/3	2/3	2/3	1/3	2/3	2/3	12/18
C57BL/6	KLH/ICFA[a]	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	—	3/3	3/3	3/3	3/3	2/3	3/3	17/18
C3H/HeJ	KLH/ICFA[a]	3/3	2/3	3/3	3/3	3/3	3/3	18/18
	—	3/3	3/3	3/3	2/3	3/3	3/3	18/18

[a] Keyhole limpet hemocyanin in incomplete Freund's adjuvant.

Table III. Incidence of PCV-2 positive serum sample numbers from PCV-2 inoculated mice[a] as detected by gel-based PCV-2 PCR analysis (number of PCV-2 PCR positive serum samples/number of mice sampled)

Line		Day post-inoculation						
		12	17	22	27	32	37	Total
BALB/c	KLH/ICFA[a]	3/3	1/3	1/3	2/3	2/3	1/3	10/18
	—	2/3	1/3	2/3	2/3	3/3	2/3	12/18
C57BL/6	KLH/ICFA[a]	2/3	2/3	0/3	0/3	3/3	0/3	7/18
	—	0/3	0/3	1/3	1/3	0/3	0/3	2/18
C3H/HeJ	KLH/ICFA[a]	1/3	0/3	0/3	3/3	1/3	0/3	5/18
	—	1/3	2/3	1/3	1/3	2/3	3/3	10/18

[a] Keyhole limpet hemocyanin in incomplete Freund's adjuvant.

mice with positive serum PCR results compared with BALB/c mice. No significant differences were noted in the number of PCR positive mice between C3H/HeJ and BALB/c mice ($P = 0.206$) or between C3H/HeJ and C57BL/6 mice ($P = 0.157$). Porcine circovirus-2 DNA was present in the tissue of 92.6% (100/108) of the PCV-2 infected mice and in serum of 42.6% (46/108) of the PCV-2-infected mice. The mean amount of PCV-2 DNA present in serum samples in each mouse line on the different necropsy days is summarized in Figure 1. The median amount of PCV-2 DNA detected was 10 689 copies per 1 mL serum (25% quartile 4872 PCV-2 DNA copies; 75% quartile 23 676 PCV-2 DNA copies). The amount of PCV-2 DNA was not significantly ($P > 0.05$) different between the mouse lines on any of the necropsy days. The ISH for PCV-2 was negative in all tissues and in all mice.

Presence of anti PCV-2 antibodies

All serum samples obtained from the non-inoculated control mice and inoculated mice were negative for anti-IgG PCV-2 antibodies as analyzed by indirect and competitive PCV-2 ELISA (data not shown).

Macroscopic lesions

No gross lesions were seen in the non-inoculated, non-vaccinated control mice. In the non-inoculated, KLH/ICF injected control mice, enlarged lymph nodes were noted. In the PCV-2 inoculated mice, enlarged lymph nodes were noted. These were most prominent in BALB/c mice on days 17 to 27. Non-stimulated PCV-2 inoculated mice appeared to have larger lymph nodes than the KLH/ICFA stimulated mice.

Microscopic lesions and presence of PCV-2 antigen

Microscopic lesions consistent with PCV-2 infection (lymphocytic depletion and histiocytic replacement of follicles) were not observed in any of the mice (control and treatment). A portion of mice in all lines and treatment groups had prominent germinal centers in their lymphoid tissues (lymph node, thymus, and spleen). No significant differences among groups were noted in lesions (prominent germinal centers) in the lymph node or thymus. Mice which were treated with KLH/ICFA and inoculated with PCV-2, had a significantly greater rate of non-reactive follicles (fewer prominent germinal centers were

seen) in the spleen compared with the following groups: untreated, PCV-2 inoculated mice ($P = 0.002$); untreated, non-inoculated mice ($P = 0.001$); and KLH/ICFA treated, non-inoculated mice ($P = 0.002$). The IHC for PCV-2 was negative on all tissues from all mice.

Discussion

The first objective of this study was to determine if mice infected with a well-characterized infectious PCV-2 isolate develop characteristic lesions of PCVAD. While previous studies have indicated that BALB/c mice are susceptible to infection with PCV-2, mice had been inoculated with low titers of PCV-2 isolates (2,4). Additionally, the PCV-2 isolates used in earlier studies had not been tested for virulence in an experimental pig model (2,4). The study herein used a genetically and pathologically well-characterized PCV-2 clone that has been used for several studies in pigs (7,11,18,19). The virus derived from the infectious PCV-2 clone has been shown to induce PCVAD in conventional pigs (8).

Differences in virulence of PCV-2 isolates have been demonstrated in the growing pig model (20,21). It was hypothesized that our well-characterized PCV-2 isolate would induce more distinct lesions in the mice than those observed in previous studies. In contrast, no characteristic microscopic lesions characteristic of PCVAD (lymphoid depletion and granulomatous lymphadenitis) were identified, and ISH and IHC stains were negative. One possible explanation may be that the mouse and pig models differ in sites of replication, pathogenesis, and manifestation of PCV-2 virulence.

Another possible explanation may be that PCV-2 transmission occurs by a different route in mice than in pigs. The intramuscular and intranasal inoculation routes used herein have been proven to be effective in pigs (7,19). In previous studies, the mice were inoculated intraperitoneally and intranasally (2,3). The most likely and logical explanation for the lack of PCV-2 associated clinical signs and lesions is that PCV-2 has limited ability to infect the mouse by 2 intramuscular and intranasal inoculations. Limited replication would explain the low level of viremia seen in this study. Since serum from each mouse was only obtained at 1 point in time (on the necropsy day) it is difficult to evaluate the dynamics of the virus load over time. Based on Figure 1, there was a slight increase in amount of PCV-2 DNA around day 17 in C3H/HeN and C57BL/6 lines, and the amount of PCV-2 appeared to remain on a constant level for the remainder of
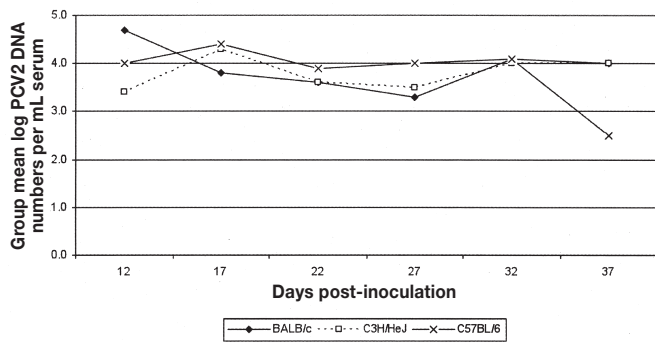
Figure 1. Mean amount of PCV-2 DNA in serum samples for each mouse line and necropsy day post PCV-2 inoculation. The data are for mice that were inoculated with PCV-2 (*n* = 6 for each necropsy day).

the experiment. This trend is similar to what is usually seen in pigs experimentally inoculated with PCV-2 where the amount of PCV-2 typically peaks between 14 and 21 days post PCV-2 inoculation, decreases slightly thereafter, but remains present over an extended period of time (6,8–10).

In addition to the low amount of PCV-2 DNA in serum samples, there was also a lack of microscopic PCV-2 associated lesions seen in this study. It has been estimated that a minimum of 10^8 PCV-2 genomes per 500 ng DNA is required to give visible staining in an IHC assay (22). Comparison of the IHC and ISH for the detection of PCV-2 found more positive cells with increased signal intensity when IHC was used in pigs (23). When the IHC and ISH assays on tissues from pigs inoculated with PCV-2 were compared, a similar observation was made (unpublished). Therefore, it is likely that the replication in the mouse occurs below the analytical sensitivity threshold of the IHC and the ISH assays used and the presence of PCV-2 DNA in tissue pools may still reflect viremia.

The results of this trial also demonstrated that mice became PCR positive on tissue pools and serum samples, but failed to induce detectable IgG antibody production. The lack of seroconversion as detected by the indirect ELISA was confirmed with a competitive ELISA. This test was used as it is not dependent on the binding capabilities of a specific anti-mouse IgG antibody; therefore, a problem with antibody binding would likely not account for the apparent negative results. The apparent lack of antibody production differs from previous studies in which seroconversion of 1/4 mice on day 14 and 3/4 mice on days 28 and 42 was reported by Kiupel et al (2) and seroconversion of 1/5 mice to PCV-1 on day 20 was reported by Quintana et al (4).

Differences in the assays used to detect anti-PCV-2 antibodies may explain the differences in results. In this trial, ELISA testing was used; previous trials determined antibody levels by immunofluorescence assay (IFA) (2) or immunoperoxidase monolayer assay (IPMA) (4). As both of these assays have subjective endpoints and some antigenic cross-reactivity with PCV-1 (24), it is possible that this may account for the differences in serology results. Additionally, an investigation of PCV-2 serological assays performed at various European and Canadian laboratories reported that results of PCV-2 IPMA and IFA testing varied highly based on the testing protocol (25).

Another objective of this experiment was to determine if there are differences in susceptibility to PCV-2 among different lines of mice. As no mice displayed gross or microscopic lesions associated with PCV-2 (even following immune stimulation with KLH/ICF) this objective could not be evaluated based on these lesions. However, a significant difference was noted in the number of mice that became PCV-2 positive in serum samples. Previous results suggest that BALB/c mice develop a highly polarized T-helper (T_H) 2 response whereas C3H/HeJ mice develop a highly polarized T_H1 response (26). In our study, C57BL/6 mice, whose immune response is between BALB/c and C3H/HeJ mice, had a lower incidence of PCV-2 viremia in serum samples compared to BALB/c mice. This may indicate that both a T_H2 and T_H1 immune response are required to combat PCV-2 infection.

The results of this study indicate that mice may not be a useful model to understand immunopathogenesis of PCV-2 associated disease and lesions. However, mice may be important in the epidemiology of PCV-2, as mice apparently replicate PCV-2 and remain viremic for extended periods and thus might serve as reservoirs for PCV-2 and play a role in PCV-2 transmission in pig populations. Further research on the susceptibility of mice to PCV-2 on and around pig farms is warranted.

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