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Research Article

HISTOPATHOLOGICAL AND IMMUNO-HISTOCHEMICAL EVALUATION OF MALE AND FEMALE REPRODUCTIVE SYSTEMS OF PORCINE CIRCOVIRUS-2 INFECTED PIGS

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ABSTRACT: Porcine Circovirus-2 (PCV-2) is an emerging swine infection responsible for significant financial losses in the global swine industry. It has a significant negative impact on reproductive performance causing abortion, stillbirth, and other anomalies. As there is limited knowledge related to the histopathology of male and female reproductive systems in PCV-2 infected pigs, the current study was designed. Swine carcasses presented for post-mortem examination with a history of respiratory distress, anorexia, diarrhoea, wasting, and paleness of the skin collected from mid of 2021 to mid of 2022 from different parts of Kerala, India, were utilised in this study. The samples were initially screened with polymerase chain reaction (PCR) and were subjected to gross and histopathological studies. Out of 65 collected samples, 10 were positive for PCV-2 by PCR. The positive sample carcasses were emaciated, had poor body condition with visible bony prominences, decreased back fat thickness, rough, long hair coat, and sunken eyes. Mild oedema and congestion were seen in the testes, epididymis, and vas deferens of the male reproductive system and on accessory reproductive glands such as the bulbourethral gland, prostate gland, and seminal vesicle. In the female reproductive system, the ovary, oviduct, and uterus had mild congestion and oedema in most cases. Histopathology of the male reproductive system revealed mild degenerative changes, haemorrhage, and congestion in all cases. The vasa deferentia showed a loss of cilia in the pseudostratified columnar epithelium. The female reproductive organs had congestion, degenerative changes, and infiltration of mononuclear cells. For further confirmation, localisation of PCV-2 antigen was done in reproductive organs with immunohistochemistry (IHC). History, gross, histopathological findings, and PCR in combination with IHC highlight the pathologic effects of PCV-2 on reproductive organs in infected pigs.

Keywords: : Porcine Circovirus-2, Testes, Uterus, Abortion.

INTRODUCTION

The main reason for the drastic decline in swine population from 10.29 million in the 19th census to 9.6 million in the 20th census (12.03%) in India (20th national livestock census) has been attributed to emerging and re-emerging pathogens [1] added with nutritional reasons like piglet anaemia [2]. A large number of these pathogens induce illnesses that have a negative influence on the swine population through morbidity and mortality. One of the main developing pathogens that significantly increase pig morbidity and mortality in India is porcine circovirus-2 (PCV-2) [3]. The pathogenesis and evolution of PCV-2 have a significant impact on the swine population, which affects the profitability of swine farmers and the protein consumption of the expanding population [4]. PCV-2 is a circular, small, single-stranded, non-enveloped DNA virus with 1766-1768 bp in size and

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belongs to the circovirus genus in the Circoviridae family [5]. The PCV-2 causes 5-30% morbidity and 50-60% mortality in the infected pigs. Infection with PCV-2 has been linked to several porcine circoviruses associated diseases (PCVAD), including exudative epidermitis and necrotizing lymphadenitis, PCV-2-associated pneumonia, porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex, granulomatous enteritis and others [6, 7]. Pigs between the ages of 8 and 16 weeks were mostly infected with PCV-2 having a severe impact on their health, including emaciation, jaundice, stunted growth, and dermatitis in the hind legs [8].

PCV-2 is believed to be a major factor in the common reproductive abnormalities in sows, such as abortions, barrenness, the delivery of mummified foetuses, and underdeveloped and dead piglets [9, 10]. Several field reports and experimental research have shown that PCV2 has the potential to be a prenatal pathogen [11]. When PCV2 spreads transplacentally during viremia or when inseminated with contaminated semen, it may affect embryos and foetuses. Vertical transmission of PCV-2 to young ones occurred during embryonic development and shedding of the virus through infected boar semen [12]. However, the gross and histopathological changes induced by PCV-2 in reproductive organs have not been studied in detail so far. Maintaining good production and producing quality offspring depend heavily on the regular functioning of reproductive organs. Any deviation from these lowers productivity and simultaneously affects the income of pig producers.

Male and female reproductive pathologies of PCV-2 infected piglets seem to be underestimated in most cases. Gross lesions were overlooked due to the lack of typical lesions. In this study, we evaluated the gross and histopathological changes in the male and female reproductive systems of PCV-2-infected piglets. The results were further validated with the help of immunohistochemistry in the reproductive organs of PCV-2-infected piglets.

MATERIALS AND METHODS

Collection of Samples

Sixty-five piglet carcasses aged up to 3 months were collected from a period between mid-2021 to mid-2022. Samples were taken from carcasses that were submitted to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India, for post-mortem investigation from various swine farms across Kerala. Samples were collected based on the clinical history of diarrhoea, respiratory distress, anaemia, wasting with visible bony prominence, presence of irregular, red-to-purple macules and papules in the skin, rough, long hair coat, and sunken eyes.

Polymerase chain reaction

The tissues showing gross lesions suggestive of PCV-2 were collected in a phosphate buffer solution and stored at -18ºC for the molecular study. Total DNA was isolated from the tissue samples suspected of PCV-2infection using Qiagen DNeasy blood and tissue kit (Catalog number: 69504). Based on earlier research [13], PCV-2 primer sequences (5'CGGATATTGTAGTCCTGGTCG3; 5'ACTGTCAAG GCTACCACAGTCA3') and PCR conditions were utilised [14]. The amplicons generated after 45 minutes of electrophoresis at 70V and 400mAV were examined on a 2 percent agarose gel. The PCR product was documented in the gel using a gel documentation system (Bio-Rad Laboratories, USA).

Gross and histopathology

Complete necropsies of the carcasses were performed and gross lesions in various organs, particularly those related to male and female reproduction, including the testes, epididymis, vas deferens, prostate, seminal vesicles, bulbourethral glands, ovary, oviduct, and uterus, as well as other visceral organs displaying gross lesions, were documented. These tissue samples were preserved in 10% neutral buffered formalin. Fixed tissue samples were undergone dehydration, clearing, paraffin impregnation, and embedding. The paraffin tissue samples were sectioned at 3-5 µm thickness and was stained with haematoxylin and eosin [15].

Immuno-histochemistry

Immunohistochemistry was used to localise the PCV-2 antigen capsid by polyclonal antibody (Invitrogen, Thermofisher Scientific, USA). The sections were rehydrated with grades of alcohol after being deparaffinized with xylene. In order to retrieve the antigen, tissue sections were heated three times at 750 W for five minutes in 0.01 M citrate buffer at pH 6.0. Non-specific antigenic sites were blocked with protein block (Abcam-ab64264) for 10 minutes at room temperature. The sections were exposed to the rabbit polyclonal anti-PCV2 primary antibody (PA5-34969, Invitrogen, Thermofisher) at a dilution of 1:200 for 24 hours at 4ºC. The sections were cleaned with

buffer before being treated for 10 minutes at room temperature with the biotinylated goat secondary antibody-ab64264 (Mouse and rabbit-specific HRP/ Dab detection IHC kit). The tissues were then covered with streptavidin horseradish peroxidase DAB solution (Avidin Biotin Complex method), incubated for 10 minutes and then rinsed with buffer for five minutes. Counterstaining was carried out using Meyer's haematoxylin. The sections were then mounted with DPX, and examined under a microscope [7].

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences version 22.0 (SPSS version 22) for Windows (SPSS IBM. Corp., Armonk NY). Non-parametric Kruskal-Wallis Test was used to analyse lesions of male and female reproductive organs. Parameters compared were degenerative changes, congestion/haemorrhage and inflammatory cell infiltration of male and female reproductive organs [16]. The histopathological changes were scored based on severity as absent (0), minimal (1), mild (2), moderate (3), and severe (4).

Results were considered significantly different if $p<0.05$. Ethical review and approval were obtained before initiation of the present study and samples were obtained from submitted cadavers for post-mortem examination.

RESULTS AND DISCUSSION

Detection of PCV-2 by PCR

Out of 65 collected samples, 10 samples were positive for PCV-2 by PCR. Carcasses of 5weaned pigs with 3 males and 2 females and 5 nursery pigs with 2 males and 3 females were PCV-2 positive by PCR out of 10 positive samples (Fig. 1). The PCR was targeted on the viral nucleocapsid gene (ORF-2) which amplified 481 bp [13]. Most of the positive animals displayed clinical symptoms, including respiratory distress, muscle wasting, anaemia, diarrhoea, rough hair coat, and chronic skin ulcers [17].

Gross lesions

Examination of gross lesions revealed that the most positive cases had irregular, red-to-purple macules, and papules in the skin, primarily on the hind legs and perineal area [8,18] Mild oedema and congestion were seen in the testes, epididymis and vas deference of the male reproductive system (Fig. 2a). Accessory reproductive glands such as the bulbourethral gland, prostate gland and seminal vesicle showed congestion and oedematous changes (Fig. 2b). In the female reproductive system, the ovary, oviduct and uterus had mild congestion and oedema in most of the cases (Fig. 2c and 2d). Additionally, other visceral organs also showed severe gross lesions. Lymphoid organs showed severe oedema, lymphadenopathy and congestion [14]. Lungs were non-collapsing, oedematous, and congested with localised haemorrhages with granulomatous pneumonia. The intestinal mucosa was thickened and haemorrhagic with mesenteric oedema, mesenteric lymph node enlargement, catarrhal enteritis and intense congestion or pale/yellowish discolouration was seen in the liver in all the positive cases. Congestion and multifocal severe petechial haemorrhages were seen in the kidneys. There was pale yellowish discoloration of the surface and renal papillae. There was evidence of curdled milk in the stomach and the mucosa had mild to severe oedema [19].

Histopathology

Histopathology of the present study indicated that in the male reproductive system, testes and epididymis were having mild degenerative changes, haemorrhage, mononuclear infiltration, and congestion in five cases (Fig. 3a, 3b, 3c and 3d). Loss of cilia in the pseudostratified columnar epithelium of vasdeferens in some cases (Fig. 3e). Accessory reproductive glands such as the prostate, bulbourethral glands, and seminal vesicles showed mild oedema and congestion in three cases (Fig. 3f, 3g and 3h). In female reproductive organs, ovaries and uterus had congestion, degenerative changes, and infiltration of mononuclear cells. Vacuolation and degenerative changes in endometrial glands were present in the uterus in all cases (Fig. 4a, 4c and 4d) [11]. In the oviduct, congestion and haemorrhages were noticed in five cases (Fig. 4b). PCV-2 had a lot of effects on the ovary through proliferation but no prominent lesions were detected in the female reproductive system [20]. Other visceral organs also showed severe microscopic lesions. Vasculitis in the renal pelvis, severe fibrino-necrotising glomerulitis, multifocal haemorrhages, congestion and infiltration of mononuclear cells were observed in the kidneys of infected animals (Fig. 5a). Emphysema, haemorrhage, congestion, interlobular septal oedema, vasculitis, and infiltration of mononuclear cells were evident in the lungs (Fig. 5b).

Lymphoid depletion in the follicular area with necrotising lymphadenitis and histiocytic infiltration was seen in mesenteric lymph node in most of the cases (Fig. 5c). Soft palate tonsils also showed multi-

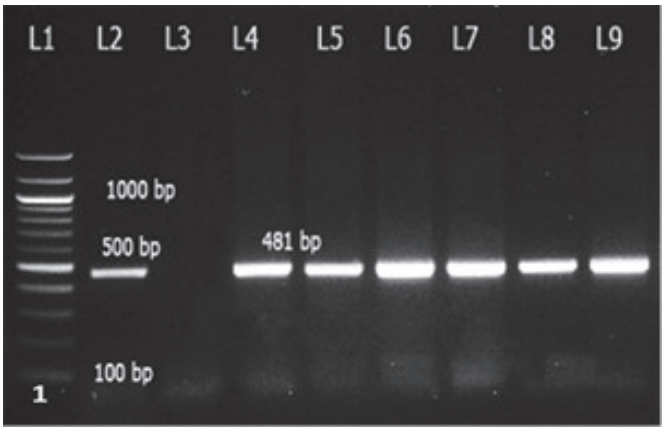


Fig. 1. Agarose gel electrophoresis picture showing 481 bp PCR amplified product of PCV-2. (Lane 1-DNA ladder lane 2-positive control lane 3-negative control lanes 4 to 9 - positive samples).

focal haemorrhages, severe lymphoid depletion and infiltration of lympho-histiocytic cells [7]. Nearly all of the cases in this study had moderate to severe histiocytic infiltration and moderate to severe lymphoid depletion around the periarteriolar sheaths of the spleen. Botryoid inclusion bodies were observed in the spleen [8]. Infiltration of inflammatory cells and haemorrhagic gastritis, ulceration and necrosis of the mucosal layer in the stomach was evident. Congestion and haemorrhages in between myocardial fibers were present. Additionally, degeneration of the myocardium and infiltration of lymphocytes and macrophages were observed in the heart. Lympho-histiocytic cells around the portal triadand hepatocellular necrosis were noted on the liver (Fig. 5d). Also, segmental or circumferential fibrinoid vasculitis was observed in kidneys, lungs and spleen [21].

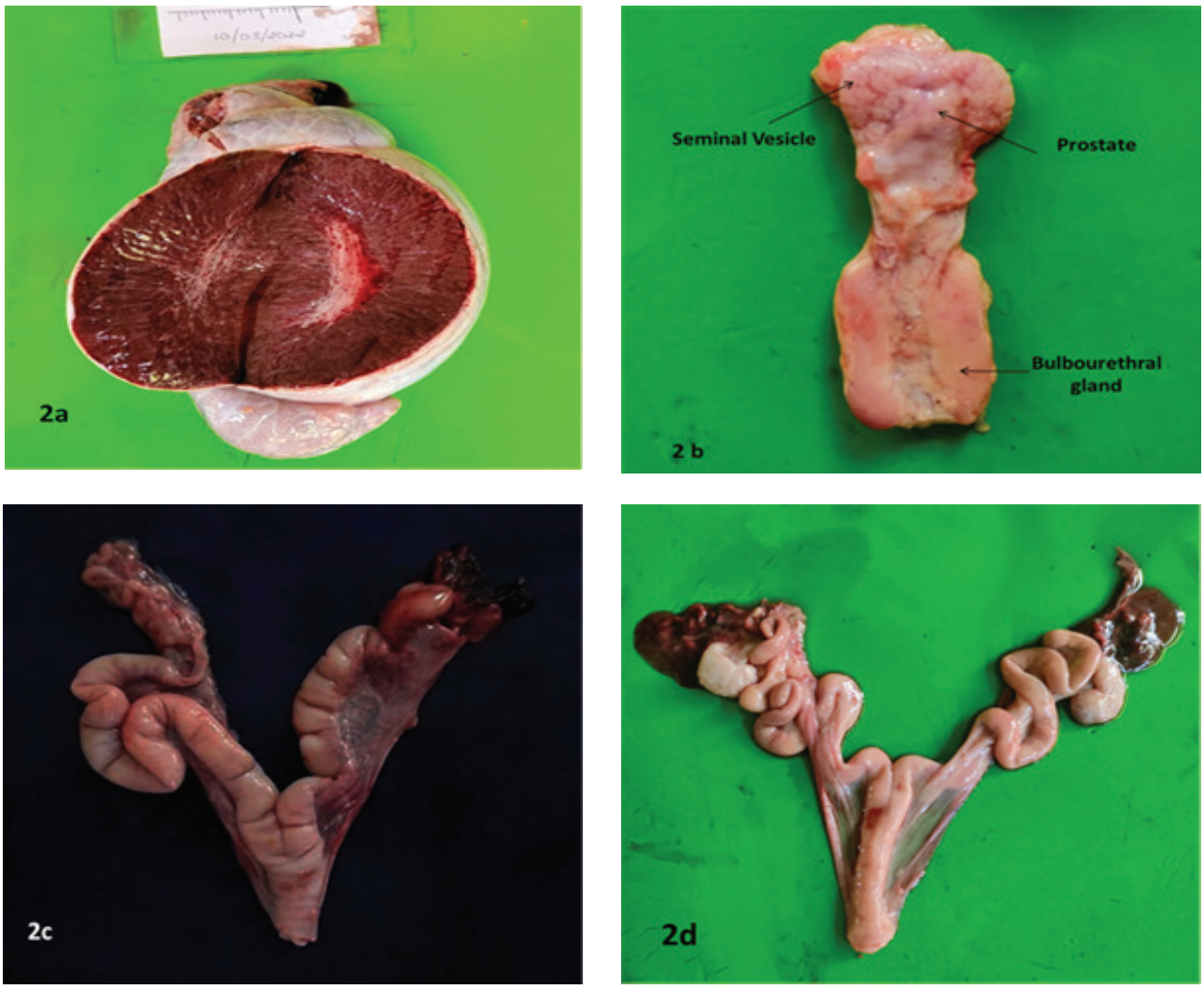


Fig. 2. Gross lesions of male and female reproductive organs. [2a. Congestion and oedema in testes, 2b. Mild oedema in prostate, seminal vesicle and bulbourethral glands, 2c. Severe congestion and oedema in ovary, oviduct and uterus, 2d. Mild congestion and oedema in ovary, oviduct and uterus].

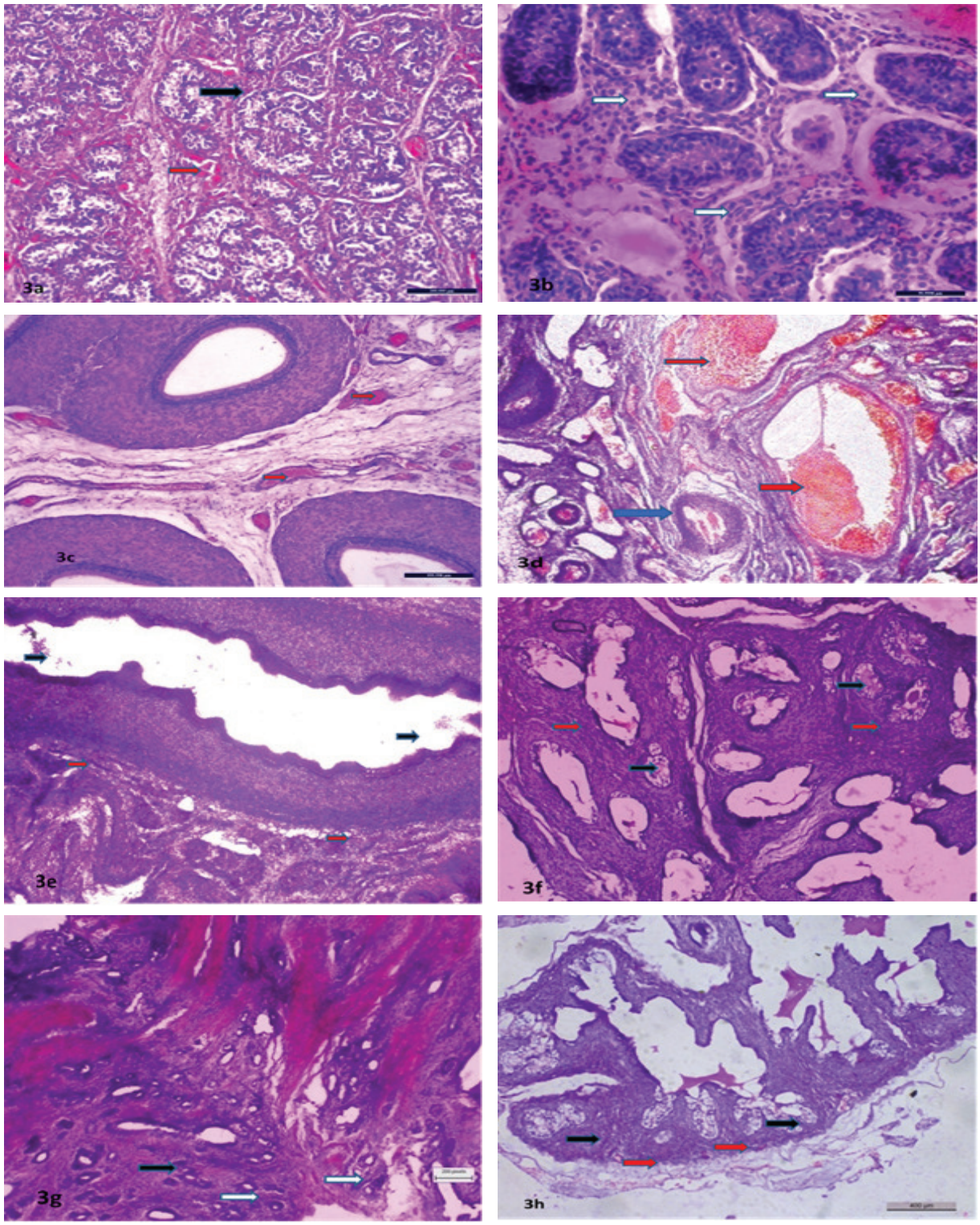


Fig. 3. Histopathology of male reproductive system. [3a. Mild mononuclear infiltration in the interstitium (black arrow), haemorrhage and congestion of blood vessels (red arrow) in testes (H&E X 100),3b. Congestion and infiltration of lympho-histiocytic cells (arrow) in testes (H&E X 400), 3c. Moderate congestion in epididymis (H&E X 200), 3d. Severe congestion (red arrow) and mononuclear infiltration (blue arrow) in epididymis (H&E X 200),3e. Mild congestion (red arrow) and loss of cilia (black arrow) in vas deferens (H&E X 100), 3f. Mild congestion (red arrow) and oedema (black arrow) in bulbourethral gland (H&E X 100),3g. Mild congestion and oedema (white arrow) in prostate (H&E X 100),3h. Congestion (white arrow) and oedema black (arrow) in seminal vesicle (H&E X 200)].

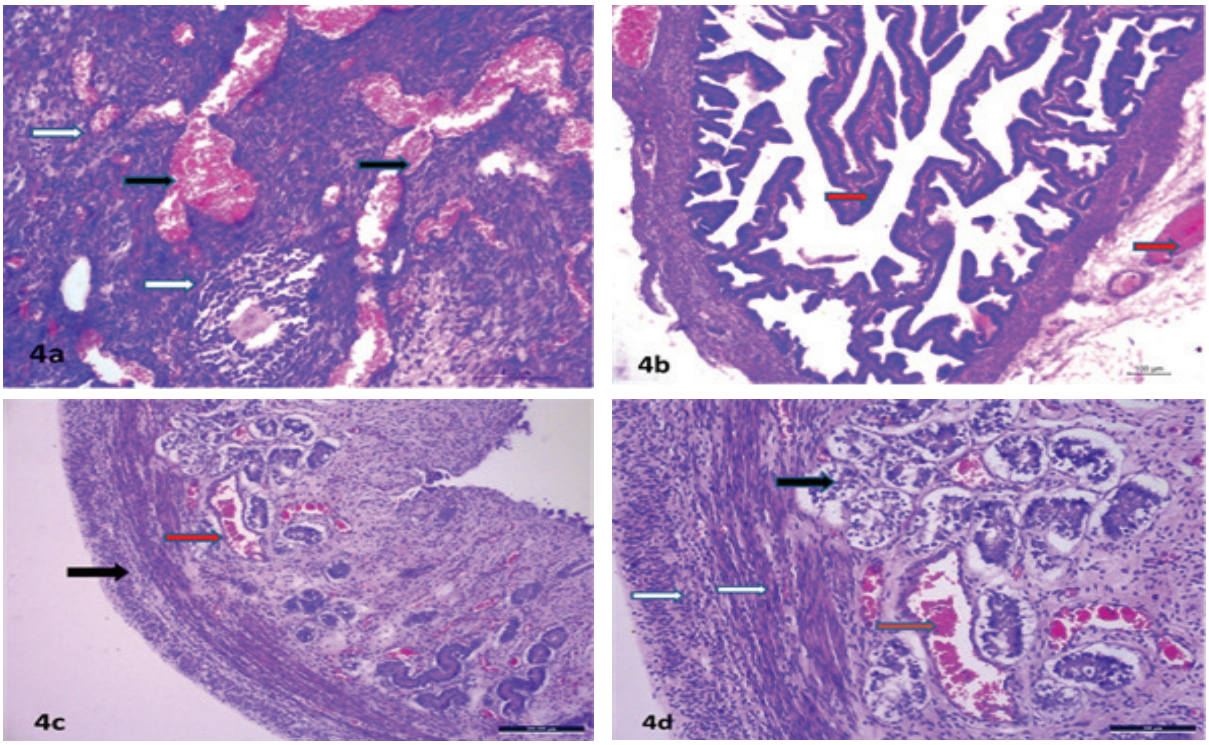


Fig. 4. Histopathology of female reproductive system. [4a. Severe congestion (black arrow) and moderate infiltration of mononuclear cells (white arrow) in ovary (H&E X 400), 4b. Moderate congestion of oviduct (arrow) (H&E X 400), 4c. Moderate congestion (red arrow) and infiltration of mononuclear cells (black arrow) in uterus (H&E X100,4d. Moderate congestion (red arrow), mononuclear cell infiltration (white arrow) and vacuolation and degeneration of endometrial glands (black arrow) in uterus (H&E X200)].

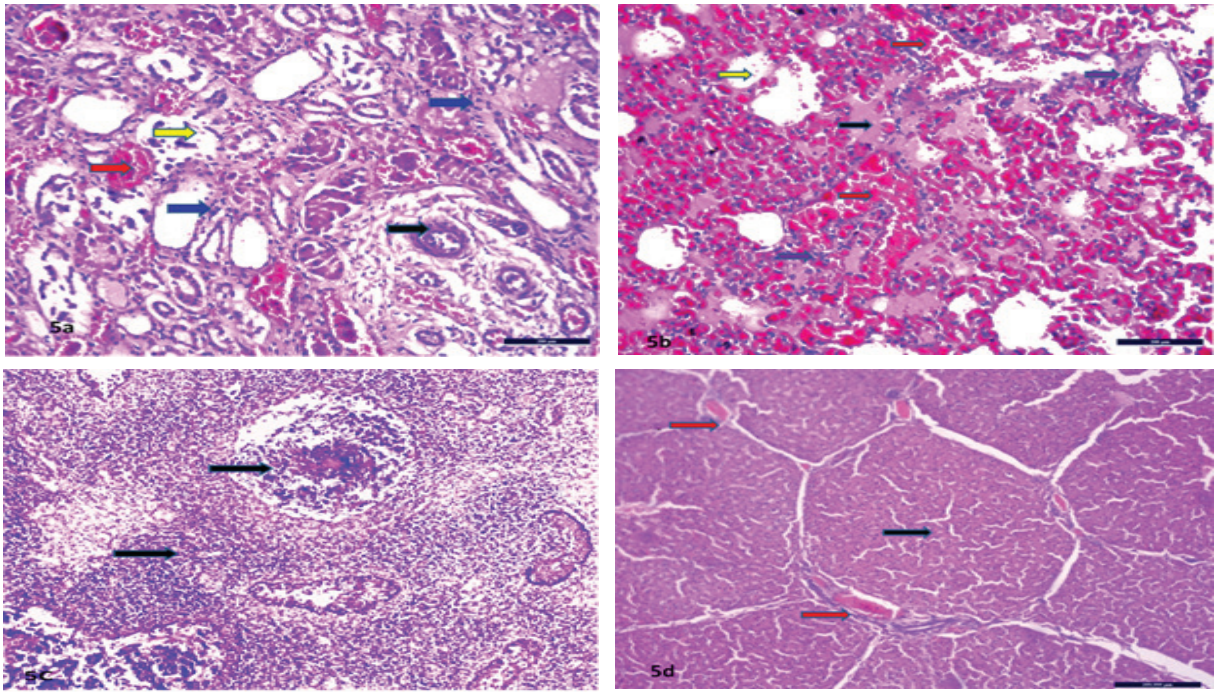


Fig. 5. Histopathology of visceral organs. [5a. Vasculitis in the renal pelvis (black arrow), severe fibrino-necrotising glomerulitis, multifocal haemorrhages, congestion (red arrow), disruption of tubules (yellow arrow) and infiltration of mononuclear cells (blue arrow) in kidney (H&E X200), 5b. Emphysema (yellow arrow), haemorrhage, congestion of alveolar capillaries (red arrow), interlobular septal oedema(black arrow), vasculitis and infiltration of mononuclear cells in the lungs (blue arrow) in lungs (H&E X200), 5c. Lymphoid depletion in the follicular area with necrotising lymphadenitis- histiocytic infiltration(arrow) and congestion in mesenteric lymphnode (H&E X 200), 5d. Lympho-histiocytic infiltration around periportal vein (red arrow) and extensive hepatocellular necrosis (black arrow) in liver (H&E X 100)].

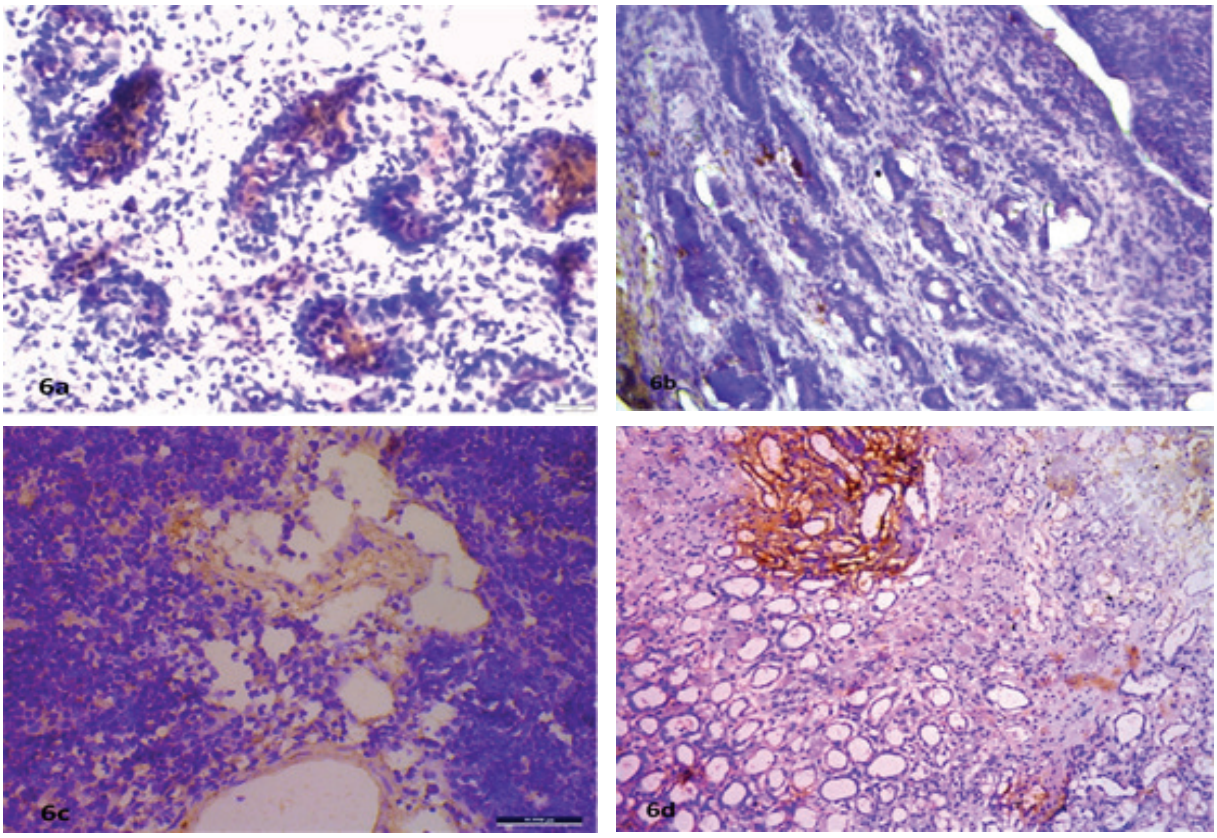
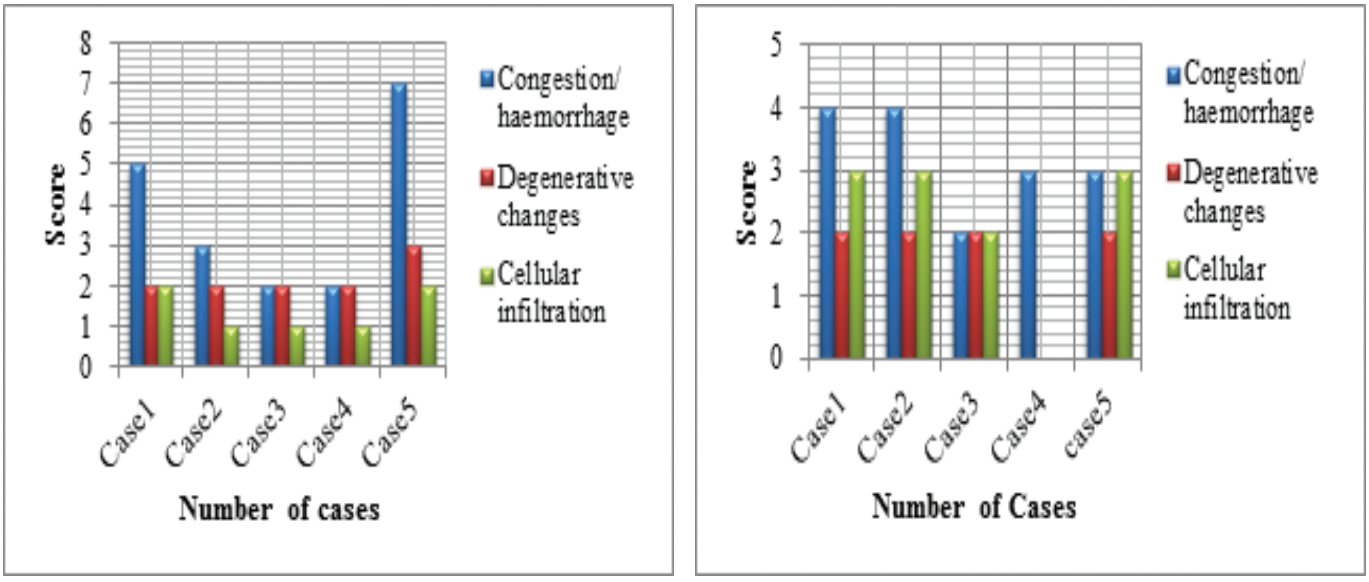


Fig. 6. Representative Immunohistochemical images of immunopositivity of PCV-2 infection. [6a. immunopositivity of testes to PCV-2 antigen appeared as brown colour (IHC X 400); 6b. Immunopositivity of uterus to PCV-2 antigen appeared as brown colour (IHC X 200); 6c. Lymph node revealed PCV2 antigen positive signals (brown) (IHC X 100); 6d. Kidney revealed PCV2 antigen positive signals (brown) (IHC X 200)].



A **B**

Fig. 7. Histopathological scoring of the reproductive system of pig. [Male: (7A), female: (7B)].

Immunohistochemistry

Using PCV-2 polyclonal antibodies, IHC was carried out to localise PCV2 virus antigen in the reproductive organs. PCV-2 antigen was successfully detected by IHC in the testes and uterus of PCR-positive tissue samples. With DAB as the chromogen, the affirmative cells displayed the expected dark brown reaction result (Fig. 6a and 6b). Various cells of these organs showed brown cytoplasmic staining as a result of PCV-2 immunopositivity. Lymphoid organs and other visceral organs such as lungs, intestine and kidney showed PCV-2 antigen by IHC (Fig. 6c and 6d). PCV-2 infections were confirmed by demonstrating IHC in conjunction with distinct microscopic features. Furthermore, lesions of male and female reproductive organs were scored are listed in Fig. 7a and 7b [15, 16].

This study mainly involves molecular and pathological studies of male and female reproductive systems of porcine circovirus-2 infected pigs. According to the results of the study, PCV-2 affects both male and female reproductive organs. It causes severe congestion, degenerative changes and lympho-histiocytic infiltration in the testes, ovary, epididymides, oviduct and uterus. Due to chronic immunological stimulation towards PCV-2, it is clear from these histopathological studies that PCV-2 has a tropism towards reproductive organs similar to other lymphoid and visceral organs. If the animal survives the infection, this might affect the future performance of the animal. Natural or artificial mating cause the transmission of the virus which will affect the healthy animals in the swine herd [12]. The inflammatory changes, congestion, haemorrhages and degenerative changes on testes and epididymis had a negative impact on reproductive performance as it may result in low semen quality and quantity which also affect the transportation of semen and finally results in sterility or infertility. In females also virus is shed on the young ones through colostrum and also affects embryonic development [22, 23]. The lesions caused by PCV-2 on female reproductive organs result in disruption of the normal physiology of these organs. The PCV-2 may disrupt ovum development, pathologic changes to the oviduct affect fertilisation, and in the uterus affect implantation that results in premature death and abortions. In the end, it leads to underdeveloped piglets, abortions, mummified foetuses, infertility and sterility. The reproductive failure caused by PCV-2 poses a serious threat to farmers who raise pigs, which lowers production and results in significant financial loss. Our findings imply that evaluating the immunopathogenesis and evolution of PCV-2 infection will further advance strategies against vaccine development in swine.

CONCLUSION

The current study was designed to evaluate the effect of PCV-2 on reproductive organs and the long-term implications. This work provides a better understanding of the vertical transmission of viruses to young animals and provides deeper insight into the virus shedding by boar semen. Therefore, this work contributes to our understanding of the effects of gross and histological lesions observed on male and female reproductive systems by PCV-2 infection to advance quicker, better control strategies to prevent reproductive disorders in the swine herds.

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