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Myeloid to Erythroid (M: E) ratio in the evaluation of bone marrow cytology of Porcine Circovirus type 2 affected pigs

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Abstract

Porcine circovirus associated diseases (PCVAD) caused by porcine circovirus type-2 (PCV-2) are emerging viral diseases with unfavourable effects on animal health and swine economy. We have a lot of information regarding the changes in the lymphoid organs and spleen in PCV-2 infected pigs whereas the reason for anaemic changes in the carcasses and the pathological effects of PCV-2 in bone marrow are still not well studied. Hence, an extensive study to identify the changes in myeloid and erythroid cells of bone marrow in PCV-2 infected pigs was carried out. Myeloid and erythroid series of cells were counted and analysed from the freshly collected bone marrow cytological smears from the PCV-2 suspected samples. Later, PCV-2 infection was confirmed by polymerase chain reaction (PCR) and characteristic histopathological findings. The PCR yielded an amplicon of ~ 481 bp product and those positive cases were selected for determining the Myeloid to Erythroid ratio (M : E ratio). However, values did not significantly differ in any of the cellular components between PCV-2 positive animals and PCV-2 negative animals which indicated that the bone marrow was not the specific target organ for PCV-2 viral infections. However, increased lympho-histiocytic and plasmacytic infiltration was noticed in both lymphoid and non-lymphoid organs. These characteristic features of PCV-2 infection could be considered as a major reason for increased proliferation of myeloid cells.

Keywords: *PCV-2, polymerase chain reaction, bone marrow M: E ratio*

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PCV-2 infection was first reported thirty years back in Canada. Later it has been reported globally in association with many disease manifestations. They are PCV-2-systemic disease (PCV-2-SD), PCV-2-reproductive disease (PCV-2-RD) and porcine dermatitis and nephropathy syndrome (PDNS), which are now recognised as porcine circovirus diseases (PCVD) or porcine circovirus-associated diseases (PCVAD) (Segalés, 2012; Correa-Fiz *et al.,* 2020). The PCV-2 infection is considered as an immunosuppressive disease with devastating effects on global swine farming (Saikumar and Das, 2019). The infected animals expressed clinical signs such as wasting, diarrhoea, unthriftiness, pallor, respiratory distress and icterus (Rossell *et al*., 1999). Lymphoid organs mainly lymph nodes and spleen are found to be extensively affected in PCV-2 infection (Segales *et al.*, 2000; Sharma *et al.,* 2010). Profound alteration of the immune system is characteristic of for PCV-2 infections. Microscopically, there was lymphoid depletion in T and B cell areas and histiocytic infiltration in lymphoid organs and presence of multinucleated giant cells (Keerthana *et al.*, 2017; Sairam *et al.,* 2019). These changes in the lymphoid organs are well documented. However, there is limited information on the changes in bone marrow in PCV-2 infection. Bone marrow is widely studied for haematopoietic and mesenchymal cells morphologies (Tadjalli *et al.,* 2013). Alteration in the cytology of myeloid and erythroid progenitor cells usually happens in viral infections. Early detection of these changes could help in management of the disease as well as its prevention and control. Hence, the present study was aimed with the objectives of evaluating bone marrow of PCV-2 infected pigs. This study can improve our current understanding of the effects of PCV-2 on bone marrow of pigs.

Materials and methods

Sample collection

A total of 39 pig carcasses suspected of PCV-2 infection were submitted to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences Mannuthy, Kerala during the period from March 2019 to November 2020 for postmortem examination. The pooled tissue samples from mesenteric lymph nodes, tonsils, lungs and bone marrow (Keerthana *et al.*, 2017) were collected and investigated. Lymphoid organs and non-lymphoid organs were collected in 10% neutral buffered formalin for histopathological evaluation (Suvarna *et al*., 2019). The PCV-2 was detected by PCR from pooled tissue samples using primers designed for the nucleocapsid gene (*ORF-2*) specific for PCV-2 (Ellis *et al.,* 1999).

Bone marrow smear examination

The bone marrow smears were collected from the epicondyle portion of the femur by paint brush technique as soon as possible after death (Reagan *et al.*, 2011). The smears were fixed using methanol and were stained by Wright-Giemsa stain. The stained smears were examined under oil immersion objective. The myeloid and erythroid ratio was estimated by counting 500 cells in total (Ryan, 2001). The data were expressed as Mean ± SEM. All results were processed using SPSS (Version 24.0 for windows, SPSS Inc., Chicago, IL, USA). The results were analysed using Student's *t*-test for comparison between PCV-2 positive and negative animals. The bone marrow smears from the apparently healthy slaughtered pigs (PCV-2 negative animals) were treated as the control group for our study. Statistical significance was considered at $p < 0.05$.

Results and discussion

The primers were selected for PCR for the region specific to the nucleocapsid gene (*ORF-2*) as per previously published reports (Ellis *et al.,* 1999; Sairam *et al.,* 2019). In PCR, the amplified products were obtained at 481 bp amplicons. In the current study, 39 samples were processed for the detection of PCV-2 by PCR, among them seven were positive for PCV-2 **(**Fig.1).

Affected animals were seen emaciated with enlarged lymph nodes. Gross lesions noticed were hydropericardium, diffusely non-collapsing lungs, pale friable liver, multifocal white areas on mucosa of tonsil or congestion and ulcerative lesions of soft palate, splenic

infarcts, swollen kidneys with foci of pallor and haemorrhages (Fig. 2 and 3) Lymphocytic depletion and histiocytic infiltration were noticed in all the lymphoid organs of PCV-2 positive animals (Fig. 5). Histiocytosis was noticed in histological sections of bone marrow (Fig. 4). Kidney revealed cloudy swelling in the renal tubules (Fig. 6). Although a significant statistical difference could not be observed in haematopoietic precursors between PCV-2 positive and negative animals (Table 1); an increase in mean myeloid and erythroid counts was observed in PCV-2 positive animals **(**Fig. 7 and 8). The mean cellular percentage of myeloid and erythroid precursors of bone marrow of pig are listed in Table 1.

The present study employed the PCR technique as a diagnostic tool to detect PCV-2 virus by using the viral nucleocapsid gene (*ORF-2*) specific PCR. Bone marrow suppression is a general manifestation in viral infection, especially in immunosuppressive infections like PCV-2 (Hansen *et al*., 2013; Pascutti *et al.,* 2016*)*. Even though immunosuppression is a feature of porcine circovirus associated diseases (Segalés, 2012), the mechanism by which PCV-2 produces immunosuppression is not clear. Here, lymphoid depletion and histiocytic infiltration were noticed in all the cases of PCV-2 affected pigs in lymphoid organs which were in agreement with the

Table 1. The mean cellular percentage of myeloid and erythroid precursors of bone marrow of pigs

Myeloid cells	Mean percentage in PCV-2 negative samples	Mean percentage in PCV-2 positive samples	Erythroid cells	Mean percentage in PCV-2 negative samples	Mean percentage in PCV-2 positive samples
Myeloblast	7.53±0.13	7.830±0.17	Rubriblast	1.10±0.17	1.40±0.24
Promyelocyte	10.75±0.30	11.25±0.65	Rubricyte	2.5±0.27	2.50±0.27
Myelocyte	12.25±0.52	9.30±0.15	Metarubricyte	6.00±0.69	2.75±0.27
Metamyelocyte	17.50±0.55	22.41±0.46	Polychromatic normoblast	21.38±0.53	13.86±0.22
			Reticulocyte	10.00±1.09	13.00±0.79
			RBC	8.07±0.81	7.486±0.25
Total	48.03±0.32	50.79±0.46	Total	49.05±0.59	41.58±0.34

The mean percentage values of the myeloid and erythroid cells in PCV-2 positive animals were 50.79 and 41.58 respectively, where as in PCV-2 negative animals were 48.03 and 49.05 respectively. We observed an increase in myeloid to erythroid ratio (M: E) in PCV-2 affected pigs. When compared with PCV-2 negative animals, the PCV-2 positive animals had reduced percentage of haematopoietic tissue in the bone marrow (Fig. 7 and 8). A numerical decrease of erythroid cells and mild increase of myeloid cells were noticed on bone marrow cytology. The M: E ratio in PCV-2 positive animals was 1.2: 1 whereas that in apparently healthy pigs was 0.9:1.

previous reports of Keerthana *et al.* (2017) and Sairam *et al*. (2019). In the present study, cases suggestive by gross and histopathological lesions were confirmed by PCR.

In the present study, carcasses of PCV-2 infected pigs were blanched, anaemic and also had an increased myeloid series of cells. The characteristic features of myeloid series of cells have been described (Cowell *et al.,* 2007). Myeloblast cells are morphologically undifferentiated cells having prominent nucleoli in the nucleus and agranular cytoplasm. Nucleoli are also visible in promyelocytes but the cytoplasm mostly has non-specific

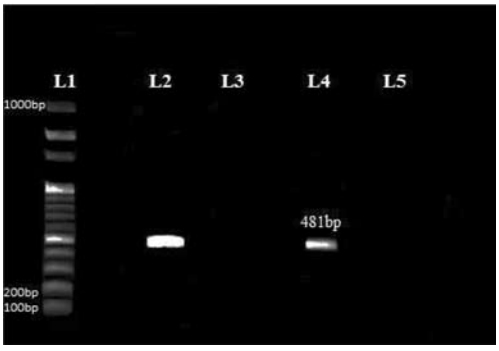

Fig. 1. Agarose gel electrophoresis picture showing 481 bp PCR amplified product of PCV-2 (lane 1-DNA ladder; lane 2-positive control; lane 3-negative control; lane 4 –positive sample; Lane 5 – negative sample)

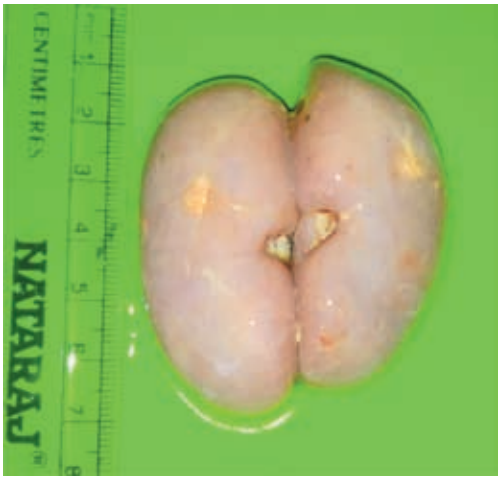

Fig. 2. Kidney showing few white areas with multi-focal pinpoint haemorrhages

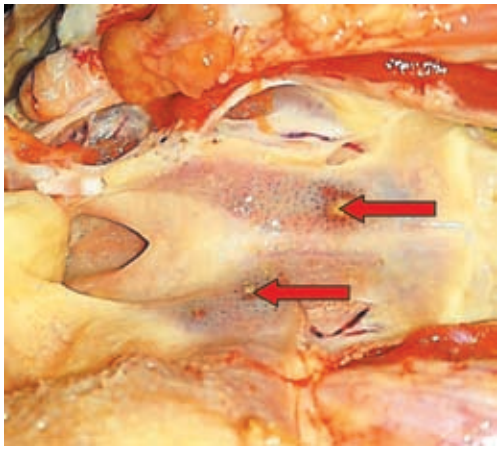

Fig. 3. Soft palate tonsil showing congestion and multi-focal ulcerative lesions (arrows)

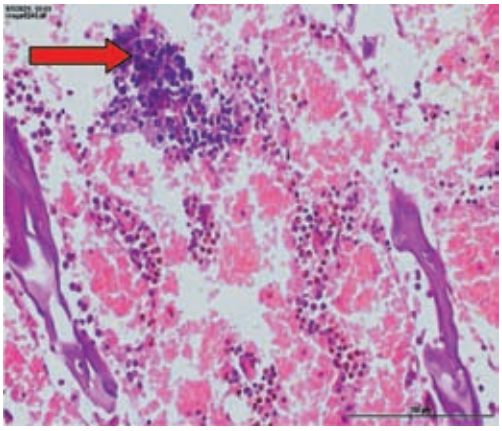

Fig. 4. Hypocellular bone marrow with areas of histiocytosis (arrow) (H&E X 100)

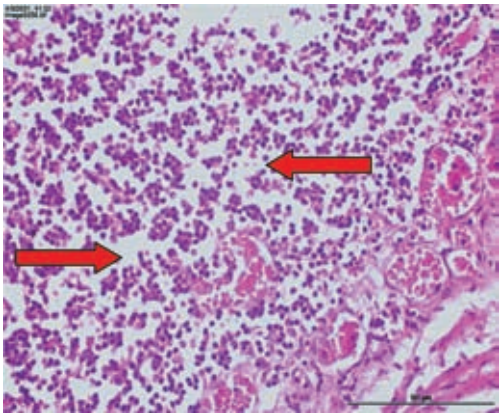

Fig. 5. Lymph node showing severe histiocytic infiltration (arrows) (H&E X 400)

granules. Promyelocyte matures and later differentiate into myelocyte. From the myelocyte stage onwards, the cells start differentiating

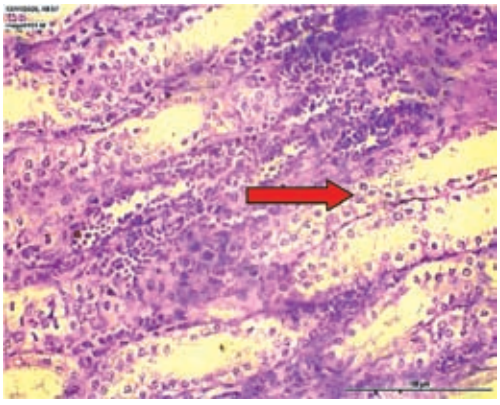

Fig. 6. Kidney showing cloudy swelling in the renal tubules (arrow) (H&E X 400)

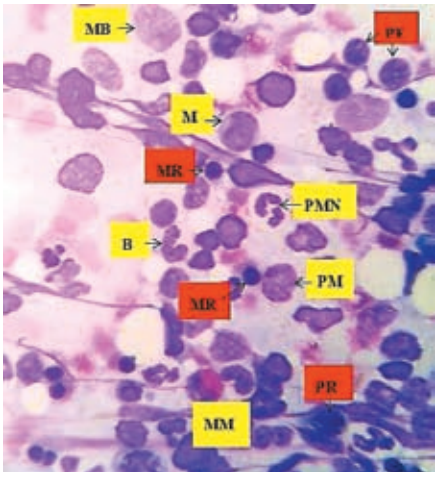

Fig. 7. Bone marrow cytological smear showing different myeloid and erythroid series of cells (Wright-Giemsa stain, 1000x). MB- Myeloblast; PM – Promyelocyte; M – Myelocyte; MM – Metamyelocyte; B- Band cells; PMN- Matured segmented neutrophil; PR – Prorubricyte; PE- Polychromatophilic erythrocyte; MR- Metarubricyte

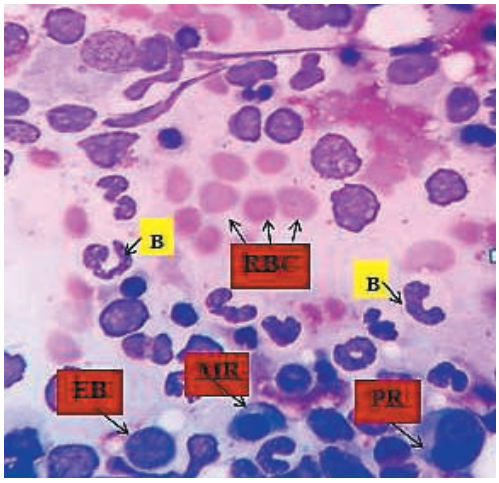

Fig. 8. Bone marrow cytological smear showing different myeloid and erythroid series of cells (Wright-Giemsa stain, 1000x) EB- Erythroblast; B- Band cells, PR – Prorubricyte, MR- Metarubricyte; RBCS-Red Blood Cells

into neutrophilic, basophilic and eosinophilic myelocytes. When the indentation of the nucleus starts to appear in the myelocyte these cells are classified as metamyelocyte. If the indentation of metamyelocyte nucleus becomes greater than half of the cell, it is called a band cell. Matured neutrophils have distinctive lobes inside the cells.

In the present study, reduced erythroid series of cells in PCV-2 infected pigs were also noticed. The characteristic features of erythroid series of cells have been described (Cowell *et al.*, 2007). The rubriblasts are the most immature form of erythroid cells, having higher nuclear cytoplasmic ratio. The cytoplasm is intensely basophilic and forms a narrow rim around the nucleus. Nucleus has clear smooth round border with one or two pale to medium blue nucleoli. In prorubricyte, the nuclear chromatin is slightly coarser, nucleolus is usually not visible and the cytoplasm forms a thick rim around the nucleus. The rubricyte has spokes of wheel appearance with extremely coarse chromatin. The colour of the cytoplasm varies from blue to bluish-red-orange to red-orange. The nucleus of metarubricytes is highly pyknotic and appears black with indistinct nuclear chromatin pattern. Polychromatophillic erythrocytes are the non-nucleated erythrocytes and are larger than the matured erythrocytes. Mature erythrocytic stage is the last stage in erythroid cell series maturation.

The increased M: E ratio could be due to decline in erythroid activity and increase in myeloid activity. An increased M: E ratio in the current study was due to reduction of erythroid cells and increase in myeloid cells in the bone marrow. Since PCV-2 has the potency to produce lesions in kidney (Rossell *et al.,* 1999), it is unable to affect erythropoiesis in infected animals. Erythroid hypoplasia of bone marrow could be related to the fact that anaemia is a common finding in PCV-2 affected cases which was in agreement with Segales *et al*. (2000).

The increase in myeloid cells might be due to their demand to encounter the PCV-2 viral infection. Increased mononuclear infiltration in the thickened alveolar septa of pneumonic lung, histiocytic infiltration in lymphoid and non-lymphoid organs also accounted for

the increased demand of myeloid cells. Bone marrow histological examination also suggested focal histiocytosis which evinced an increase in proliferation of monocyte/ macrophage lineage cells which might be due to PCV-2 infection. This fact could be related to the increase of macrophages (as histiocytic and plasmacytic cells) infiltrating in target tissues such as lymphoid and non-lymphoid organs which is characteristic of PCV-2 infection. This also could be stated as a reason for increased proliferation of myeloid cells observed in cases of PCV-2 infection (Rosell *et al*., 1999).

Conclusion

A detailed analysis to recognize the effects of PCV-2 on the myeloid and erythroid series of bone marrow based on cytological smears in pigs was carried out. The results indicated that there is an increase in M: E ratio in PCV-2 infected pigs which might be due to hypoplasia in erythroid cells and hyperplasia of myeloid cells. Hence, this study has improved the current understanding of pathological effects on bone marrow in PCV-2 infection. However, further studies are required with a greater number of samples to validate the preliminary results of our study as PCV-2 has the highest mutation rates among DNA viruses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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