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Pathological and hematological alterations in different stages of horn core carcinoma of cattle in Chhattisgarh plains

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Abstract

This research investigates the pathological and hematological characteristics of horn core carcinoma (Squamous Cell Carcinoma - SCC) in cattle within the Chhattisgarh plains. SCC is a malignant neoplasm of the stratified squamous epithelium, frequently affecting the horns of zebu cattle. Stages of horn core carcinoma are determined on the basis of gross, histopathological and hematological examination. Grossly, horn neoplasms showed unilateral cauliflower-like growths at the base. Horn core carcinoma or squamous cell carcinomas (SCCs) of horn were categorized into well, moderately, and poorly differentiated types via histopathology and immunohistochemistry. Well-differentiated SCCs exhibited severe keratinization with "cell nests" and irregular tumor islands invading the dermis. Moderately differentiated horn core carcinoma showed small keratin pearls and mitotic figures. Poorly differentiated horn core carcinoma lacked distinctive keratin pearls despite deep invasion from the primary site. Hematological examination was carried out in clinical cases of both stages of horn core carcinoma (stage 1 and 2; n=20) with control (n=20), detailed comparison was done to report hematological disturbances in subsequent stages of horn core carcinoma as a result of tumour progression and malignancy. Anemia, monocytosis and neutrophilia was evident in clinical stage 1 and prominent in clinical stage 2 of horn core carcinoma in cattle when compared to healthy control.

Keywords: Anemia, Hematology, horn core carcinoma, keratinization, monocytosis, neutrophilia

Introduction

Squamous Cell Carcinoma (SCC) represents a prevalent malignant neoplasm characterized by metastatic potential, manifesting in diverse clinical forms across various animal species and humans. The acquisition of oncogenic proliferative and metastatic capabilities is driven by the accumulation of genetic and epigenetic alterations—defined as stable, heritable modifications in gene expression and cellular function absent of primary DNA sequence changes. Pathologically, SCC is a malignancy arising from the stratified squamous epithelium of the dermis or mucosal membranes. Within various bovine populations across the globe, the most frequent and clinically significant anatomical sites associated with Squamous Cell Carcinoma (SCC) involvement typically include the horn core, the periocular structures, and various regions of the cutaneous epithelium. Among these, horn core carcinoma is generally observed to be unilateral in nature and is most frequently encountered in adult cattle within the age range of 5 to 10 years (Tyagi and Singh, 2006) [17]. In the specific geographic context of India, horn cancer affects approximately 1% of the total cattle population and

accounts for a substantial 83.34% of the total bovine tumours reported in veterinary literature (Singh *et al.*, 2005) [15]. Clinically, horn cancer is characterized as a sporadic yet highly malignant neoplasm that specifically affects the horn core epithelium; it is predominantly seen in aged zebu bullocks, while it is documented only rarely in buffaloes (Somvanshi, 1991; Kumar and Thilagar, 2000) [16, 6]. Consequently, it remains one of the most commonly encountered neoplastic conditions of major economic importance, particularly regarding the health and productivity of zebu bullocks used for agricultural labor (Udharwar *et al.*, 2008) [18].

Histopathological classification trifurcates horn core carcinoma into differentiated, moderately differentiated and undifferentiated types (Kumar *et al.*, 2023; Kumar *et al.*, 2024) [8, 9]. Well-differentiated SCCs are identified by intradermal islands of stratified epithelium featuring central cell nests as result of formation of keratin by stratified squamous cells in concentric pattern surrounded by peripheral connective tissue (Kumar *et al.*, 2025) [10]. Conversely, undifferentiated SCCs are characterized by epithelial cell aggregates arranged in cords, islands, or

trabeculae, lacking central keratinization (Sharma *et al.*, 2020) ^[14]. While squamous cell carcinoma (SCC) is known to manifest across a diverse range of anatomical locations such as the cutaneous skin, external ear, vulva, and vaginal mucosa, Ocular form of SCC (OSCC) was reported in cattle of Chhattisgarh plains by Kumar *et al.*, (2023) ^[8]. The current research investigation focuses its primary attention specifically on the most frequently encountered bovine clinical manifestations: horn core carcinoma or squamous cell carcinoma localized to the horn. The notably high incidence rate of horn cancer observed within non-descript animal groups might be largely attributed to the significantly larger and more concentrated population of these non-descript cattle residing throughout the Chhattisgarh state region. Furthermore, genetic predisposition has been identified as a well-established biological phenomenon, evidenced by the fact that horn cancer is substantially more prevalent in purebred zebu cattle characterized by long horns as compared to various non-descript breeds, while the condition remains virtually absent among exotic breeds and their subsequent hybrid offspring.

Anemia and eosinophilia represent some of the most prevalent and consistent hematological findings among cattle exhibiting significant neoplastic growth and tumor progression, particularly as observed in the more advanced clinical cases of horn cancer (Somvanshi 1991) ^[16]. The condition often manifests as a chronic non-regenerative anemia; such anemia is a frequent and characteristic systemic manifestation of malignant neoplastic growth and sustained physiological stress. Furthermore, the neutrophilia observed in these clinical presentations would more likely represent a direct manifestation of severe, secondary bacterial infections complicating the primary malignancy. Due to the combined effects of systemic bacteremia, active hemolysis, and internal hemorrhage observed in these subjects, significantly higher mean values for neutrophils and monocytes were observed in tumour affected bovines compared to healthy populations (Kumar *et al.*, 2025) ^[10]. To gain comprehensive and detailed insights into the various hematological alterations—such as anemia, monocytosis, neutrophilia, and eosinophilia—that occur in clinical cases of horn core carcinoma, and to perform a thorough comparison with the physiological parameters of healthy cattle reared under traditional conditions in the rural areas of the Chhattisgarh plains, this systematic study was undertaken with the following specific objectives:

1. Detailed hematological examination in clinical cases and suspected cases of horn core carcinoma in cattle of Chhattisgarh plains.
2. Comparative analysis of hematological parameters of cases with healthy control and two different stages of horn cancer.

Hematological alterations in epithelial tumours of Cattle in Chhattisgarh plains

Detailed hematological analysis of Squamous cell carcinoma (SCC) reveals that only a limited number of comprehensive studies have been conducted in the past to facilitate a thorough comparison of hematological alterations in bovines specifically affected by this condition. Somvanshi (1991) ^[6] performed extensive research on the

occurrence of horn cancer in Indian cattle and carried out a detailed hematological analysis of the affected subjects. The investigation revealed that anemia was the most prevalent and consistent finding among cattle exhibiting significant neoplastic growth and tumor progression. Furthermore, a marked condition of eosinophilia was frequently observed in the more advanced clinical cases of horn cancer. Gomes *et al.* (2012) ^[3] also conducted research focusing on the specific hematological alterations associated with SCC of the horn. Their scientific findings were largely consistent with the presence of chronic non-regenerative anemia, reinforcing the consensus that anemia is a frequent and characteristic systemic manifestation of neoplastic growth in these animals. The observed neutrophilia in these cases would more likely represent a systemic manifestation of the severe and underlying bacterial infections often associated with ulcerated lesions.

Material and Methods

This study serves as an extension of previous research on the pathology and diagnosis of squamous cell carcinoma in bovines, conducted at the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Anjora, Durg (Chhattisgarh). A total of twenty blood samples from clinical cases of cattle with horn core carcinoma and squamous cell carcinoma were collected in accordance with IAEC recommendations to investigate potential hematological alterations. Blood samples of 2-4 ml were collected twice from the jugular vein in fortnight intervals to compare the blood parameters. into K3EDTA (tripotassium ethylenediaminetetraacetic acid) vials. Hematological data from the previous studies were utilized as a comparative reference. The haematological examination was carried out in the Clinical Pathology lab at the Teaching Veterinary Clinical Complex, Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Anjora, Durg (Chhattisgarh). A total of 40 blood samples (clinical cases (stage 1 & 2) + control) were collected to compare hematological parameters, with a specific focus on monocytes, which were found to be significantly increased in clinical cases of bovine horn core carcinoma. The principles of hematological examination were followed as per Brown (1993) ^[1] and Weiss and Wardrop (2010) ^[19]. Blood samples were processed using both wet chemistry (Trivitron fully automated Vet Hematology Analyzer-3 part) and dry chemistry (Idexx 3-part semi-automatic vet hematology analyzer) methods. The results were optimized, and the accurate values obtained after optimization and approximation were recorded.

Gross Pathology

Neoplastic growths clinically suspected to be squamous cell carcinoma (SCC) at the horn region were subjected to detailed gross examination. During stage 1, representing the early clinical phase of horn core carcinoma, a distinct lateral bending of the affected horn was frequently observed (Fig. 1a & 1b), alongside the presence of large, irregular tissue masses situated at the horn base. In the majority of recorded cases classified as stage 2, prominent cauliflower-like growths were observed emerging from the basal region of the horn (Fig. 1c). Regarding their physical properties, most

of the identified tumours exhibited a soft, friable consistency, while a smaller proportion demonstrated a firm consistency with solid nodular growth patterns (Fig. 1d). In advanced presentations, large cauliflower-like and severely ulcerated growths were noted, often accompanied by active bleeding at the horn base and deep invasion into the underlying anatomical tissues. In these specific cases of horn cancer, the external surfaces of most tumours appeared significantly rough and verrucous, displaying very poor

demarcation from the surrounding healthy areas. The cut surfaces of the excised tumours presented a varied color palette, ranging from whitish yellow and grayish white to grayish red or light brown, typically featuring widespread internal hemorrhages and localized areas of necrosis. Furthermore, ulcerative masses characterized by a foul-smelling purulent discharge were also documented in several advanced cases of horn cancer.

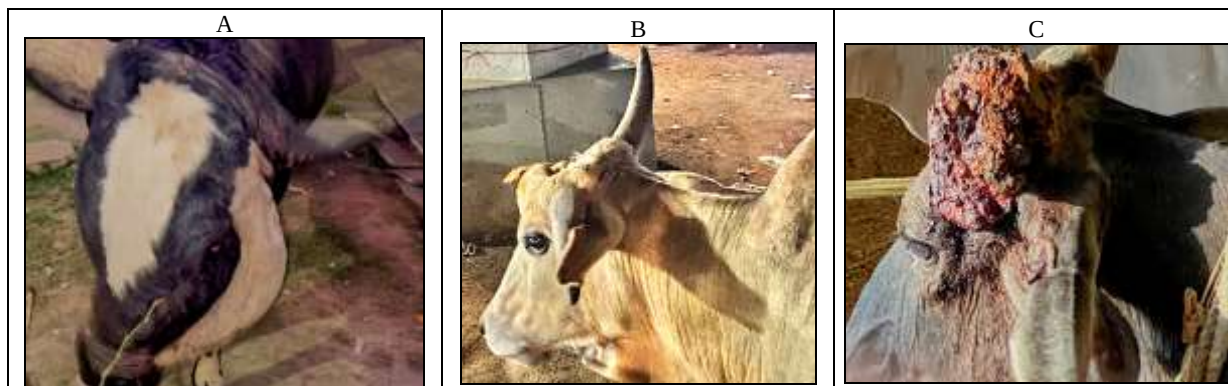


Fig 1: a & b (Lateral bending of the left horn): early stage, Fig. 1c) Stage 2 (Cauliflower like verrucous growth at the base of left horn): late stage

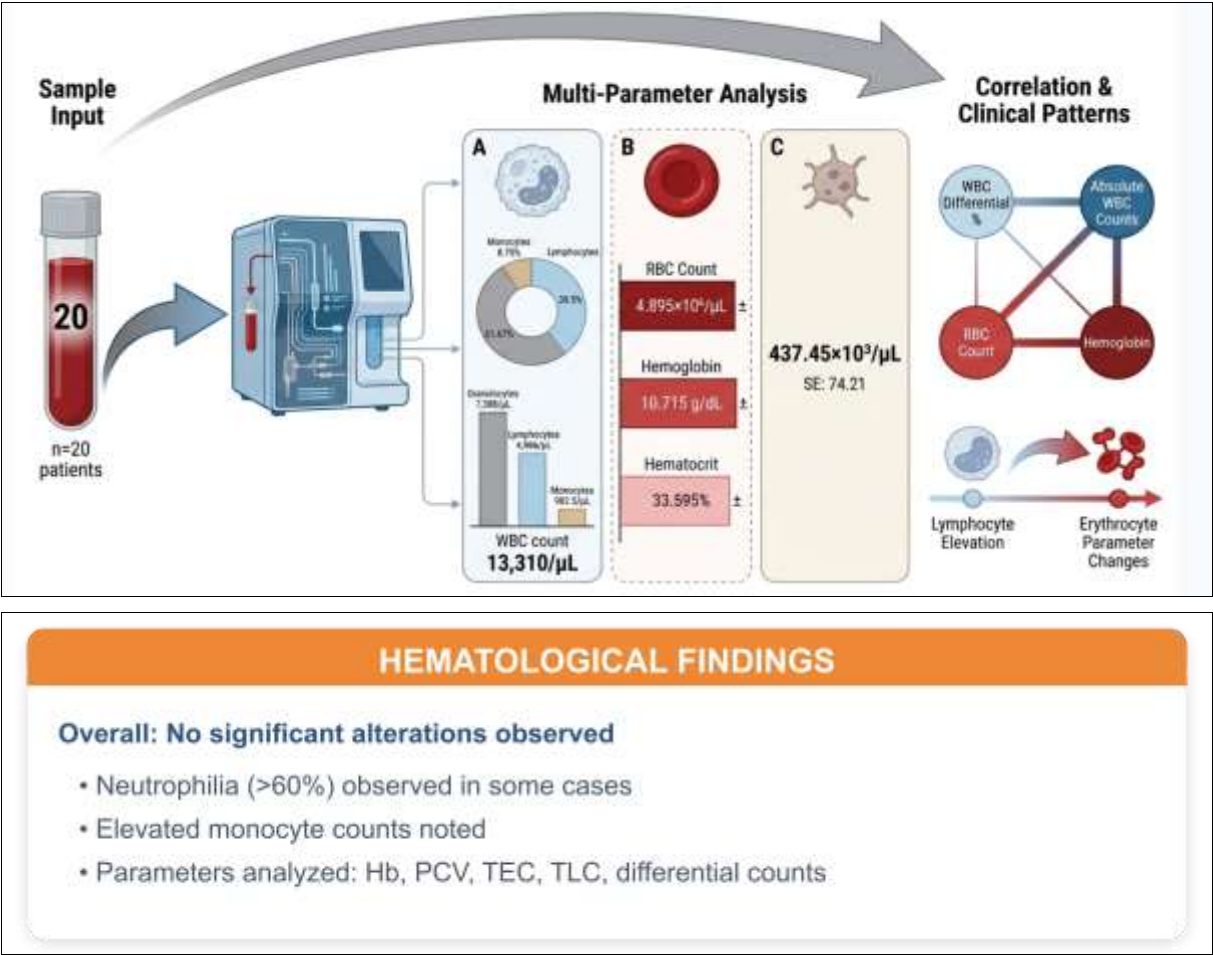
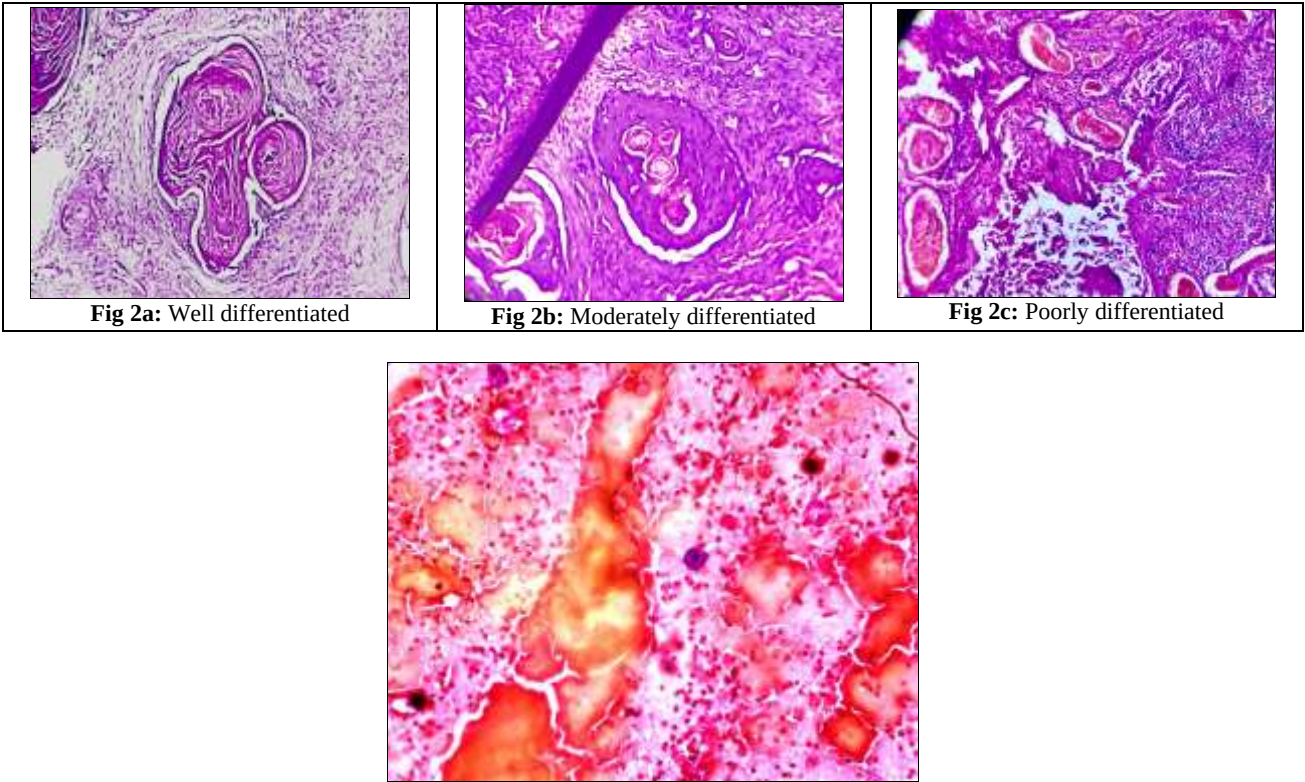


Fig 1: d) verrucous (wart like) neoplastic buds observed in the transition stage from stage 1 to stage 2 of horn core carcinoma.

Histopathological examination:

Histopathological examination of horn SCC revealed characteristic epithelial pearls with mineralization and concentric keratinization, forming "cell nests" (Fig. 2a). Most cases showed central keratin deposition, including large keratin cysts and microcysts in well-differentiated tumors. While layered pattern of keratinization was present, some cases were identified as non-keratinizing SCC. Irregular tumor islands invaded the dermis, displaying atypical squamous cells and numerous mitotic figures.

Observations included epidermal hyperplasia with hyperkeratosis and mononuclear cell infiltration. Cases ranged from poorly differentiated SCC with deep invasion and absence of epithelial pearls (Fig. 2c) to moderately differentiated SCC containing epithelial pearls (Fig. 2b). Notably, some cases lacked distinctive keratin pearls (Fig. 2c). Additional findings included severe hemorrhages and inflammatory cell infiltration, likely linked to angiogenesis, alongside ballooning degeneration in tumor cells.





Graph 1: Hematological examination with detailed comparison of cases (n=20; 1st stage) and control (n=20); graphical analysis (Source: Grabstrack)

Haematological examination

The detailed observation and statistical analysis of the mean $\pm$ standard error (SE) values derived from twenty individual bovine blood samples, focusing specifically on critical markers such as Hemoglobin (Hb), packed cell volume (PCV), Total Erythrocyte Count (TEC), and Total Leukocyte Count (TLC), have been systematically presented in Table 1.

Stage 1: Within the scope of the previous investigation, the specific hematological parameters recorded in stage 1 of horn core carcinoma included Hemoglobin (Hb) at 8.40 ± 0.18 g/dl, Packed Cell Volume (PCV) at $26.20 \pm 0.61\%$, Total Erythrocyte Count (TEC) at 6.94 ± 0.33 Million/cmm, and Total Leukocyte Count (TLC) at 8.65 ± 0.29 Thousand/cmm. Furthermore, the Differential Leukocyte Count (DLC) results revealed percentages of Neutrophils at $42.00 \pm 3.51\%$, Lymphocytes at $40.4 \pm 0.71\%$, Monocytes at $9.2 \pm 2.47\%$, Eosinophils at $1.00 \pm 0.15\%$, and Basophils at $0.40 \pm 0.12\%$, while the Platelet count was measured at

285.6 ± 2.442 thousand/cu.mm., as illustrated in Graph1; Table 1. The comprehensive findings indicated that there were no statistically significant overall hematological alterations observed in the bovines diagnosed with squamous cell carcinoma; however, notable variations were detected in specific instances, such as neutrophilia (greater than 60%) occurring in 60% of cases (n=12) involving horn core carcinoma. Additionally, an abundance of lymphocytes (45%) was identified in neoplasms of the horn—which were subsequently confirmed as squamous cell carcinoma (SCC) through histopathological examination—and abnormal monocyte values were recorded in two specific cases of horn cancer, likely attributed to underlying conditions such as bacteremia, hemolysis, and hemorrhage (Kumar *et al.*, 2025). Ultimately, significantly higher mean values for both neutrophils and monocytes were observed in bovines affected by tumours (stage 2; graph 2) when directly compared with the physiological baselines of healthy bovine subjects.

Monocytes number (Stage 2)
1200
1300
700
500
2300
2100
490
1300
200
500
900
600
140
3200
300
200
250
270
1800
1400
Mean: 982.5
SE: 187.6374934

Table 1: Haematological parameters of clinical cases, control of horn core carcinoma or squamous cell carcinoma of horn in cattle (Kumar *et al.*, 2025)

S. No.	Haematological parameters	Affected bovine early stage 1; day1 (n=20)	Observed values (range)	later stages stage 2; day 15 (n=20)	Observed values Range (stage 2)	Control (n=20) (Mean± SE)
1.	Haemoglobin (g/dl)	8.40 ± 0.18	7.6-9.6	10.71±0.84	2.6-15.9	10.55 ± 0.58
2.	PCV (%)	26.20 ± 0.61	22.4-35.0	33.59 ± 2.82	9.8-60.9	32.50 ± 2.37
3.	TEC (Million/cmm)	6.94 ± 0.33	7.0-8.0	4.89 ± 0.47	0.97-8.3	7.00 ± 0.63
4.	TLC (Thousand/cmm)	8.65 ± 0.29	6.77-10	13.3 ± 1.76	4.6-31.8	8.00 ± 1.26
5.	DLC: Neutrophils (%)	42.00 ± 3.51	25-62	51.67± 5.71	6-88.4	24 ± 2.84
	Lymphocytes (%)	40.4 ± 0.71	37-45	39.52 ± 5.84	7.6-90	60 ± 4.74
	Monocyte (%)	9.2 ± 2.47	01-28	8.75 ± 1.68	2-26.9	5.00 ± 1.58
	Eosinophils (%)	1.00 ± 0.15	00-01	1.15 ± 0.18	0.95-1.45	6.00 ± 1.89
	Basophils (%)	0.40 ± 0.12	00-01	0.50 ± 0.08	0.05-0.90	1.00 ± 0.31
6.	Platelet count (PLT) - (thousand/cu.mm.)	285.6 ± 2.442	283-300	437.45 ± 74.21	27-1152	488 ± 74.6

Discussion

Gross findings such as dorso-lateral bending of the affected horn were supported by Kalim *et al.*, (2021) ^[4]. Verrucous, cauliflower like tumorous growth on the base of horn in second stage of horn core carcinoma were supportive of the findings of Giri *et al.*, (2011) ^[2]; Sharma *et al.*, (2020) ^[14]; Kumar *et al.*, 2023, 2025) ^[8, 10]. Histopathological findings such as keratin deposition, including large keratin cysts and microcysts in well-differentiated tumors, epidermal hyperplasia with hyperkeratosis and mononuclear cell infiltration were in accordance with Pugliese *et al.*, (2014) ^[12]; Sharma *et al.*, (2020) ^[14]

Based on the comparison of mean haematological parameters across the three groups ('Early Stage 1', 'Later Stage 2', and 'Control'), here are the key observations:

Haemoglobin (g/dl)

- **Early Stage 1:** 8.40 g/dl
- **Later Stage 2:** 10.71 g/dl
- **Control:** 10.55 g/dl

Observation: Haemoglobin levels are notably lower in the early stage of affection compared to both later stages and the control group. In later stages, the levels recover to be similar to the control group.

PCV (%)

- **Early Stage 1:** 26.20%
- **Later Stage 2:** 33.59%
- **Control:** 32.50%

Observation: Similar to haemoglobin, PCV is lower in the early stage and increases in the later stage, reaching levels comparable to the control group.

TEC (Million/cmm)

- **Early Stage 1:** 6.94 Million/cmm
- **Later Stage 2:** 4.89 Million/cmm
- **Control:** 7.00 Million/cmm

Observation: Total Erythrocyte Count (TEC) is slightly lower in the early stage compared to control, but significantly drops in the later stage which indicates anemia in the affected. This is an interesting contrast to Haemoglobin and PCV which increased in later stages.

TLC (Thousand/cmm)

- **Early Stage 1:** 8.65 Thousand/cmm
- **Later Stage 2:** 13.30 Thousand/cmm
- **Control:** 8.00 Thousand/cmm

Observation: Total Leukocyte Count (TLC) is elevated in the later stage of affection compared to both early stage and control, suggesting an ongoing immune response or inflammation.

DLC: Neutrophils (%)

- **Early Stage 1:** 42.00%
- **Later Stage 2:** 51.67%
- **Control:** 24.00%

Observation: Neutrophil percentage is significantly higher in both early and later stages of affection compared to the control group, with the highest percentage observed in the later stage. This indicates a strong inflammatory response.

DLC: Lymphocytes (%)

- **Early Stage 1:** 40.40%
- **Later Stage 2:** 39.52%
- **Control:** 60.00%

Observation: Lymphocyte percentage is lower in both affected stages compared to the control group, suggesting a relative lymphopenia or a shift in the leukocyte differential count due to neutrophilia.

DLC: Monocyte (%)

- **Early Stage 1:** 9.20%
- **Later Stage 2:** 8.75%
- **Control:** 5.00%

Observation: Monocyte percentage is higher in both affected stages compared to the control group, which can also be indicative of an inflammatory or immune response.

DLC: Eosinophils (%)

- **Early Stage 1:** 1.00%
- **Later Stage 2:** 1.15%
- **Control:** 6.00%

Observation: Eosinophil percentage is significantly lower in both affected stages compared to the control group.

DLC: Basophils (%)

- **Early Stage 1:** 0.40%
- **Later Stage 2:** 0.50%
- **Control:** 1.00%

Observation: Basophil percentage is lower in both affected stages compared to the control group.

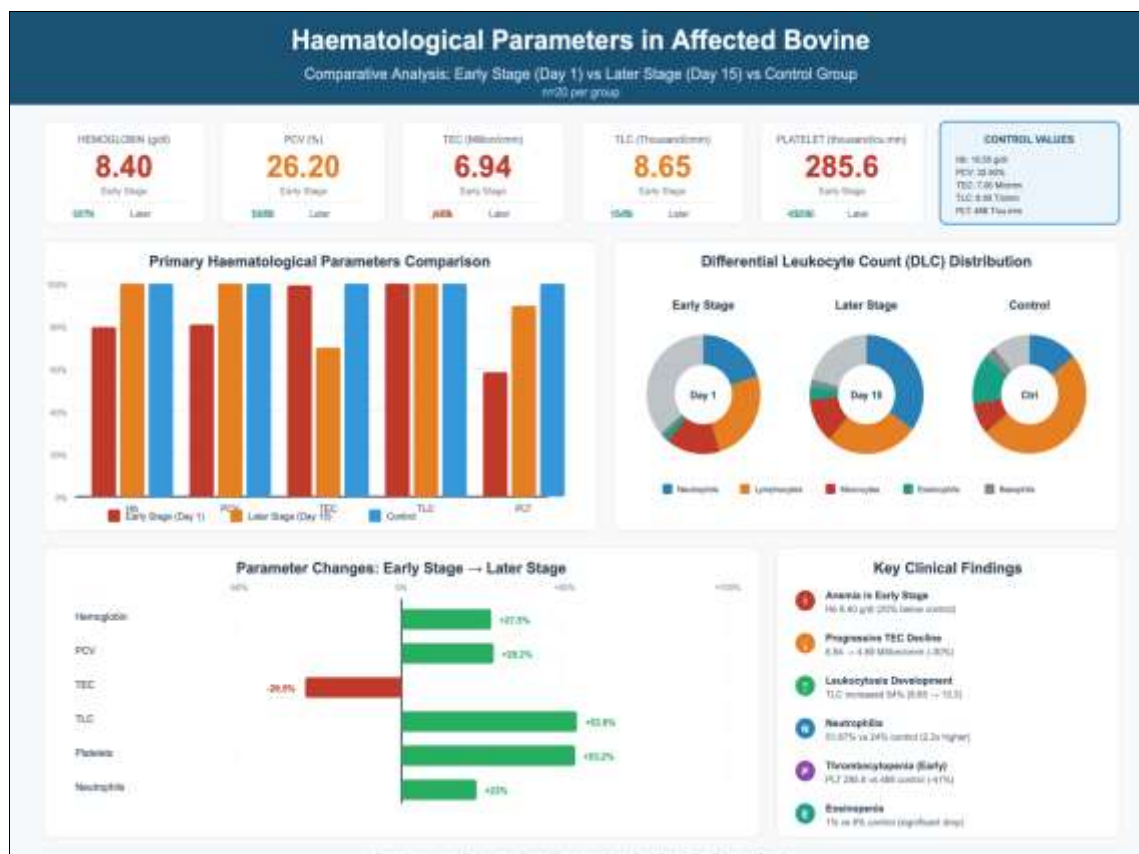
Platelet count (PLT) - (thousand/cu.mm.)

- **Early Stage 1:** 285.60 thousand/cu.mm.
- **Later Stage 2:** 437.45 thousand/cu.mm.
- **Control:** 488.00 thousand/cu.mm.

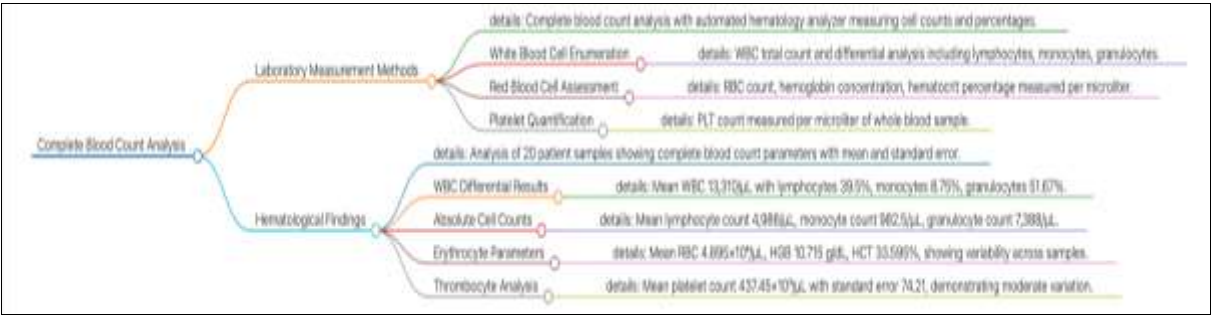
Observation: Platelet count is lowest in the early stage, increases in the later stage, but remains slightly below the control group.

The affected bovine show distinct haematological changes. In the early stage, there is evidence of anemia (lower Haemoglobin and PCV) and a shift in white blood cell differential (higher Neutrophils, lower Lymphocytes, higher Monocytes, lower Eosinophils and Basophils). In the later stages, Haemoglobin and PCV tend to recover, but TEC significantly drops. TLC and Neutrophil percentages remain elevated, indicating persistent inflammation or infection. Lymphocyte, Eosinophil, and Basophil percentages remain lower than control. Platelet counts also show a trend of recovery from early to later stages but do not fully reach control levels. These findings suggest a dynamic haematological response to the affection, with initial signs of anemia and a strong inflammatory leukocyte response that persists or evolves into later stages.

There is a notable scarcity of scientific literature regarding the specific hematological alterations and blood profile changes associated with squamous cell carcinoma (SCC) in bovine species. Despite this limited pool of existing data, the clinical findings observed in the current investigation, particularly the occurrence of neutrophilia, were found to be in close agreement and consistent with the results previously documented by Gomes *et al.* (2012) [3]. Furthermore, although a significant presence of eosinophilia was reported by Somvanshi (1991) [6] specifically in advanced clinical cases of horn cancer, the findings of the present research study did not observe or record any instances of eosinophilia in any of the examined cases involving neoplasms of the horn or the ocular region. Khurshid (2024) [5] reported a significant decrease in hemoglobin concentration and total lymphocyte numbers, which was notably coupled with a marked increase in the neutrophil count that extended well beyond the established normal reference range. For the purposes of clinical comparison, the standard normal reference values were cited in accordance with the physiological benchmarks set by Latimer and Duncan (2011) [11] and Schalm *et al.* (1975) [13]. It is important to note that no significant deviations or clinical abnormalities were observed in the remaining hematological parameters assessed during the study. Collectively, these findings strongly suggest a potential imbalance in the overall blood cell composition, specifically characterized by an elevated presence of neutrophils, which are typically indicative of an acute inflammatory response and a systemic stress-related reaction within the organism.



Graph 2: Haematological alterations observed at Stage 2 (later stage; gross features like cauliflower like growth, verrucous and friable); graphical analysis; (Source: Grabstrat).



Pathological Findings
Clinical Progression

- **Stage 1 (Early):** Characterized by lateral bending of the affected horn and the emergence of irregular tissue masses at the horn base.
- **Stage 2 (Advanced):** Manifests as prominent, friable, "cauliflower-like" verrucous growths. These tumors are often severely ulcerated, prone to hemorrhage, and exhibit poor demarcation from surrounding tissue.
- **Histopathology:** Tumors are classified into well, moderately, and poorly differentiated types. Key diagnostic features include epithelial pearls, concentric keratinization ("cell nests"), and irregular tumor islands invading the dermis. Advanced cases often show severe necrosis, inflammation, and atypical mitotic figures.

Hematological Alterations

The study compared hematological parameters between healthy cattle and those at different stages of horn core carcinoma. Key observations include:

- **Systemic Indicators:** While overall hematological

values showed variation, significant trends were identified in tumor-affected bovines compared to healthy controls.

Key Findings

- **Neutrophilia and Monocytosis:** Significantly higher mean values for neutrophils and monocytes were observed in affected cattle, likely resulting from secondary bacterial infections, active hemolysis, and internal hemorrhage associated with ulcerated lesions.
- **Anemia:** Consistent with previous literature, chronic non-regenerative anemia is a frequent systemic manifestation of tumor progression.
- **Leukocyte Dynamics:** Total Leukocyte Count (TLC) increased significantly in Stage 2 (13.3 ± 1.76 thousand/cmm) compared to Stage 1 (8.65 ± 0.29 thousand/cmm) and healthy controls (8.00 ± 1.26 thousand/cmm).
- **Neutrophil Shift:** Neutrophil percentages rose from 42.00% in Stage 1 to 51.67% in Stage 2, compared to a baseline of 24% in healthy cattle.

Summary Table: Hematological Parameters

Parameter	Stage 1 (Mean $\pm$ SE)	Stage 2 (Mean $\pm$ SE)	Control (Mean $\pm$ SE)
Hemoglobin (g/dl)	8.40 $\pm$ 0.18	10.71 $\pm$ 0.84	10.55 $\pm$ 0.58
PCV (%)	26.20 $\pm$ 0.61	33.59 $\pm$ 2.82	32.50 $\pm$ 2.37
TEC (Million/cmm)	6.94 $\pm$ 0.33	4.89 $\pm$ 0.47	7.00 $\pm$ 0.63
TLC (Thousand/cmm)	8.65 $\pm$ 0.29	13.3 $\pm$ 1.76	8.00 $\pm$ 1.26
Neutrophils (%)	42.00 $\pm$ 3.51	51.67 $\pm$ 5.71	24.00 $\pm$ 2.84
Monocytes (%)	9.2 $\pm$ 2.47	8.75 $\pm$ 1.68	5.00 $\pm$ 1.58

Conclusion

Horn core carcinoma in cattle population in Chhattisgarh plains is a progressive malignancy. Lateral bending of affected horn (stage 1), Cauliflower like growth at base of horn (stage 2) is most evident gross pathological finding. Concentric keratinization is most prominent histopathological finding of this study. Anemia, monocytosis and neutrophilia are most significant hematological findings with comparison of different stages of horn core carcinoma. The study confirms that while specific hematological values fluctuate, neutrophilia and monocytosis are reliable indicators of systemic stress and secondary infection in advanced clinical stages. Histopathological examination remains the definitive method for confirming the diagnosis of SCC through the identification of keratinized cell nests and epithelial pearls.

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Consent for publication: All authors have granted their formal consent for the publication of this manuscript.

Ethics approval and consent to participate: This research was conducted in strict accordance with the guidelines established by the Institutional Animal Ethics Committee (IAEC). All experimental procedures were performed in compliance with the recommendations of the IAEC. However, no laboratory or experimental animals were utilized in this research.

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