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Patho-epidemiological and Immunohistochemical Study of Squamous Cell Carcinoma in Cattle and Buffaloes

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ABSTRACT

Background: Study was conducted to investigate the etiopathogenesis of squamous cell carcinoma (SCC) in cattle and buffalo through pathoepidemiological, histopathological, immunohistochemical and molecular analysis.

Methods: A total of 64 tissue samples of SCC were collected between December 2024 and December 2025. Immuno histo chemistry (IHC) was performed to detect immunoexpression of pan cyto keratin (PCK), proliferating cell nuclear antigen (PCNA), zonula occludens-1 (ZO-1), E-cadherin, Vimentin, FOXA1, p120, GATA3, γ-H2AX and p63. Polymerase chain reaction (PCR) was performed to detect papillomavirus.

Result: Out of 64 cases, ocular tumors were the most prevalent, followed by horn cancer (34.38%) and vulvar tumors (9.37%). Cattle accounted for 87.5% cases, with crossbred Holstein-Friesian showing the highest occurrence. SCC was most frequently observed in females and in animals aged 7-10 years. Histopathologically, most tumors were well differentiated (WD) (70.31%). Immunohistochemically, SCCs exhibited strong expression of PCK, PCNA, p120, γ-H2AX and p63, while ZO-1 showed moderate expression. Upregulation of vimentin and downregulation of E-cadherin indicated epithelial-mesenchymal transition (EMT), whereas increased γ-H2AX expression suggested enhanced DNA double-strand damage in poorly differentiated tumors. PCR confirmed the presence of papillomavirus in four (6.25%) SCC cases.

Key words: Bovine, Immunohistochemistry, Papillomavirus, Pathoepidemiology, Squamous cell carcinoma.

INTRODUCTION

Cutaneous tumors are the most frequently diagnosed neoplasms in veterinary medicine (Goldschmidt and Goldschmidt, 2016). SCC is the most common malignant epithelial neoplasm in livestock and can arise at multiple anatomical sites *viz.* eye, horn, vulva, teat, udder, perineum and skin. In India, SCC accounts for approximately 80% of reported neoplasms in cattle and 3% in buffaloes (Somvanshi, 1991). Several risk factors have been implicated in the development of the tumor, such as chronic ultraviolet (UV) radiation, non-pigmented skin, lack of hair, chronic irritation, trauma, breed predisposition, age and sex (Velavan *et al.*, 2025).

Immunohistochemistry was performed using a panel of biomarkers to evaluate different biological aspects of SCC, including PCK for epithelial origin, PCNA for cellular proliferation, ZO-1, E-cadherin and p120-catenin for cell adhesion and epithelial integrity, vimentin for EMT, γ-H2AX for DNA double-strand break, p63 for squamous differentiation and FOXA1 and GATA3 as transcription factors (Mestrinho *et al.*, 2015; Karakurt *et al.*, 2021). A limited number of studies have evaluated immunohistochemical biomarkers associated with proliferation, EMT and genomic instability in SCC in a single cohort.

Papillomaviruses are strongly associated with papillomas and SCCs across multiple species. However, the exact mechanisms involved in carcinogenesis remain poorly understood. This limits the understanding of tumor behavior, progression and diagnostic biomarker utility in farm animals.

The objectives of the present study were to investigate the pathoepidemiological and pathomorphological

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characteristics of SCC in cattle and buffaloes, characterize histopathological lesions, evaluate immunoexpression of selected biomarkers and determine the presence of papillomavirus using PCR.

MATERIALS AND METHODS

Study period and location

The research work was conducted at the Department of Veterinary Pathology, COVSAH, KU, Sardarkrushinagar, Gujarat between December, 2024 and December, 2025.

Sample collection

A total of 87 suspected samples were examined. Histopathological evaluation confirmed 64 cases as SCC, while 23 tissue growths were diagnosed as non-neoplastic and excluded from further analyses. Among 64 cases, 22 horn cancer (HC), 36 ocular SCC (OSCC) and 6 vulvar SCCs (VSCC) were noted (Supplementary Material 1). The tissue specimens were collected from 1) Veterinary Clinical Complex, COVSAH, KU, Sardarkrushinagar 2) Anchorwala Ahinsadham, Mundra and 3) Jalaram Gaushala, Bhabhar. Tissue was collected in 10% neutral buffered formalin (NBF) for histopathological analysis and in sterile containers for molecular analysis without any preservatives and stored at -20°C as soon as possible.

Histopathology

All collected tissues were fixed in 10% NBF, subjected to standard processing. The entire tissue processing was accomplished with automatic tissue processor and subjected to hematoxylin and eosin (HandE) staining according to standard protocol of Luna (1968). SCCs were graded according to the Modified Anneroth's grading system (Table 1).

Immuno histo chemistry (IHC)

Histopathologically confirmed slides were subjected to immunohistochemical examination with various biomarkers using standard protocol of Raval *et al.* (2018). The information regarding primary antibody used in this study were mentioned in Table 2. After TBS rinsing, sections were incubated with Envision rabbit/mouse reagent conjugated with peroxidase (Dako, Denmark) for 30 minutes. Chromogenic reaction was visualized using 3,3'-diaminobenzidine (DAB) and counterstained with Harris-hematoxylin, dehydrated, cleared and mounted.

Immunohistochemical scoring was carried out by a semi-quantitative scoring system by assessing the percent of positive immunoreactive cells *i.e.* for PCK (Baghla *et al.*, 2012; Kumar *et al.*, 2025), for PCNA (Kumaraguruparan *et al.*, 2006), for ZO-1 (Upadhaya *et al.*, 2022), for E-cadherin

Table 1: Modified anneroth's grading system.

Morphological parameters	Histologic grading of malignancy of tumor cell population		
	Points		
	1	2	3
Degree of keratinization	High (>50% of cells)	Moderate (20-50% of cells)	Minimal (<20% of cells)
Nuclear pleomorphism	Little (>75% normal cells)	Moderate (50-75% normal cells)	Extensive with bizarre nuclei
Number of mitoses per high power field	2-3	4-6	>6
Pattern of invasion	*In-situ* carcinoma	Invagination into surrounding structure	Extensive infiltration with lymphnode metastasis
		Grade-I (WD): Sum of points should be 3-6	
		Grade-II: (MD): Sum of points should be 7-9	
		Grade-III: (PD): Sum of points should be 10-12	

Table 2: Details of Primary antibody used in IHC.

Antibody	Source	Clone/Isotype	Dilution
PCK	Dako, Denmark	AE1-AE3	1:100
PCNA	Dako, Denmark	PC10	1:400
ZO-1	ABclonal technology	A0659	1:500
E-cadherin	BD Transduction laboratories	36/E-Cadherin	1:100
Vimentin	Thermo scientific	V9	1:200
FOXA1	Thermofisher/Invitrogen	3A8	1:100
p120	BD Transduction Laboratories	98/pp120	1:100
GATA3	Sigma-Aldrich	L50-823	1:50
γ-H2AX	Cell signaling technology	S139	1:100
p63	Dako, Denmark	DAK-p63	1:50

Antigen-retrieval: Tris-EDTA buffer (pH-9.0) in pressure cooker; Primary antibodies were incubated for 1 hour at room temperature.

Supplementary Material 1: Details of samples collected (n=64).

Sample ID	Species	Breed	Age(Years)	Sex	Site of growth
BoHC1	Bovine	ND	7.5	Male	HC
BoHC2	Bovine	ND	8	Female	HC
BoHC3	Bovine	ND	8	Female	HC
BoHC4	Bovine	ND	10	Female	HC
BoHC5	Bovine	ND	8.5	Female	HC
BoHC6	Bovine	Kankrej	8.5	Male	HC
BoHC7	Bovine	HF cross	10	Female	HC
BoHC8	Bovine	HF cross	8.5	Female	HC
BoHC9	Bovine	HF cross	9	Female	HC
BoHC10	Bovine	HF cross	8	Male	HC
BoHC11	Bovine	HF cross	9	Male	HC
BoHC12	Bovine	Kankrej	11	Female	HC
BoHC13	Bovine	Kankrej	11	Male	HC
BoHC14	Bovine	Kankrej	8	Male	HC
BoHC15	Bovine	Kankrej	9	Male	HC
BoHC16	Bovine	ND	10	Male	HC
BoHC17	Bovine	Gir	7.5	Female	HC
BoHC18	Bovine	Gir	9	Female	HC
BoHC19	Bovine	Gir	8	Male	HC
BuHC1	Bubaline	Mehsana	9	Female	HC
BuHC2	Bubaline	Mehsana	9	Male	HC
BuHC3	Bubaline	Banni	11	Male	HC
BoEC1	Bovine	HF cross	11	Female	Ocular
BoEC2	Bovine	Kankrej	10	Female	Ocular
BoEC3	Bovine	ND	8	Female	Ocular
BoEC4	Bovine	HF cross	7	Female	Ocular
BoEC5	Bovine	ND	11	Female	Ocular
BoEC6	Bovine	ND	11	Male	Ocular
BoEC7	Bovine	HF cross	9.5	Female	Ocular
BoEC8	Bovine	HF cross	7	Female	Ocular
BoEC9	Bovine	HF cross	8	Female	Ocular
BoEC10	Bovine	HF cross	10	Female	Ocular
BoEC11	Bovine	ND	12	Female	Ocular
BoEC12	Bovine	HF cross	10	Female	Ocular
BoEC13	Bovine	HF cross	6.5	Female	Ocular
BoEC14	Bovine	Kankrej	11	Female	Ocular
BoEC15	Bovine	HF cross	12	Female	Ocular
BoEC16	Bovine	Kankrej	8	Female	Ocular
BoEC17	Bovine	HF cross	9	Female	Ocular
BoEC18	Bovine	HF cross	8	Female	Ocular
BoEC19	Bovine	ND	8.5	Female	Ocular
BoEC20	Bovine	HF cross	9	Female	Ocular
BoEC21	Bovine	HF cross	12	Male	Ocular
BoEC22	Bovine	HF cross	9.5	Male	Ocular
BoEC23	Bovine	HF cross	10	Male	Ocular
BoEC24	Bovine	ND	8	Female	Ocular
BoEC25	Bovine	Kankrej	9	Female	Ocular
BoEC26	Bovine	Kankrej	11	Female	Ocular
BoEC27	Bovine	HF cross	10	Female	Ocular
BoEC28	Bovine	HF cross	9	Female	Ocular

Supplementary Material 1: continue...

Supplementary Material 1: continue...

BoEC29	Bovine	Kankrej	7	Female	Ocular
BoEC30	Bovine	Kankrej	8	Male	Ocular
BoEC31	Bovine	Gir	9	Female	Ocular
BoEC32	Bovine	Gir	8	Female	Ocular
BuEC1	Bubaline	Mehsana	10	Female	Ocular
BuEC2	Bubaline	Mehsana	9	Female	Ocular
BuEC3	Bubaline	Banni	12	Female	Ocular
BuEC4	Bubaline	Banni	9	Female	Ocular
BoVC1	Bovine	HF cross	8	Female	Vulvar
BoVC2	Bovine	Kankrej	8	Female	Vulvar
BoVC3	Bovine	HF cross	6	Female	Vulvar
BoVC4	Bovine	HF cross	8	Female	Vulvar
BoVC5	Bovine	Kankrej	9	Female	Vulvar
BuVC1	Bubaline	Banni	8.5	Female	Vulvar

Note: BoHC: Bovine Horn Cancer, BuHC: Bubaline Horn Cancer, BoEC: Bovine Eye Cancer, BuEC: Bubaline Eye Cancer, BoVC: Bovine Vulvar Cancer, BuVC: Bubaline Vulvar Cancer, HC: Horn Cancer.

Table 3: Specifications of primers.

Particular/Target	Primer sequence(5′- 3′)	Product size (bp)	References
Quality assessment of extracted DNA using *IGF-1* gene	(F)CTCACTGTCACTGCTAAA	225	(Moore *et al.*, 2000)
	(R)AAGAAATCACAAAAGCAGC		
Papilloma virus FAP59	5′TAACWGTIGGICAYCCWTATT3′	478	(Forslund *et al.*, 1999)
FAP64	5′CCWATATCWVHCATITCICCATC3′		

(Pinho *et al.*, 2007), for p120 (Rajeev *et al.*, 2024), for γ-H2AX (Dörtbudak and Yener, 2025) and for p63 (Mestrinho *et al.*, 2015). Strong nuclear immunoreactivity for FOXA1 and strong nuclear staining in ≥10% neoplastic cells were considered as positive to GATA3 biomarker (Machado *et al.*, 2020).

Molecular detection of oncogenic virus

DNA extraction

The DNA isolation from tissues was accomplished using the commercially available DNeasy Blood and Tissue Kit manufactured by QIAGEN-GmbH, Germany according to the manufacturer's instructions. All DNA was subjected to quality evaluation using highly conserved mammalian *IGF-1* gene (Table 3).

Detection of *Papillomavirus*

Primer specific to papillomavirus were adopted from Forslund *et al.* (1999) (Table 3). Reaction mixture was prepared containing 10 µL of PCR mastermix, 0.8 µL of each primer, 2.4 µL template DNA and 6 µL PCR grade water. PCR was performed in thermal cycler (The Veriti® Applied Biosystems) according to protocol mentioned in Table 4. The detection of amplified products were performed in 1.5% Agarose gel at 85 voltages for 40 minutes and the DNA bands were visualized using Dual LED Blue/White Light Transilluminator and photographed.

RESULTS AND DISCUSSION

Incidence

Current study reported that OSCC was the predominant tumour, followed by HC and VSCC (Table 5) and found similarity with an earlier study (Parmar *et al.*, 2024). In cattle 56 cases were recorded while in buffaloes only 8 cases were found, that were similar to the findings revealed by Reddy *et al.* (2025). Among HC, 19(86.36%) were noticed in cattle whereas 3(13.64%) were diagnosed in buffaloes. Similarly OSCC and VSCC were reported higher in cattle as compared to buffaloes.

Highest incidence was noted in HF-crossbred cattle (44.64%) followed by Kankrej (25.00%), Non-descript (21.43%) and Gir (8.93%); found compatible with Yeruham *et al.* (1999) and Parmar *et al.* (2024). In buffaloes, equal distribution was reported among Mehsana (50%) and Banni breed (50%).

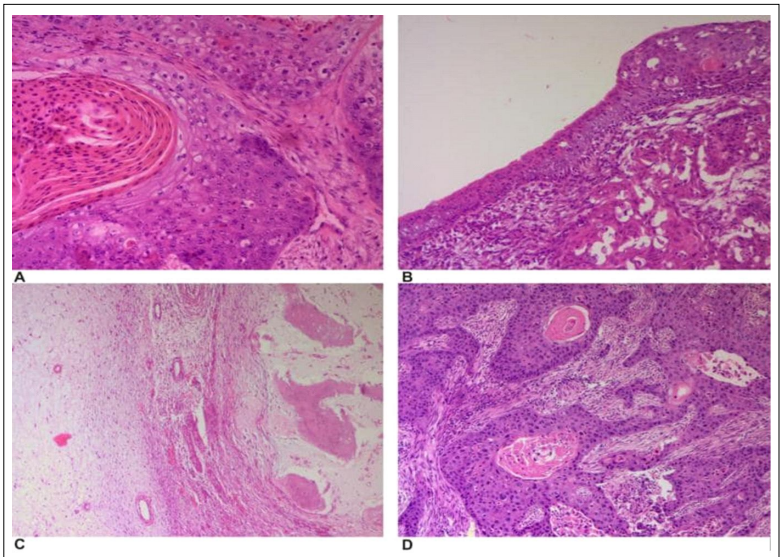
Highest incidence was observed in the 7-10 years age group(n=47), followed by <7 years (n=13) and the least in >10 years(n=4), which was comparable to Dave *et al.* (2025). However, Udharwar *et al.* (2008) reported higher occurrence in >10 years. There was equal incidence of HC among male and female. Higher occurrence of bovine OSCC was noted in females (86.11%) and were in agreement with Kalim *et al.* (2021) and Nithya *et al.* (2022).

Table 4: Protocol for PCR.

Steps	*IGF-1* gene (Quality assessment)			*Papillomavirus*		
	Temp.	Time (Min.)	Cycle	Temp.	Time (Min.)	Cycle
Initial denaturation	94°C	5	Single	94°C	10	Single
Denaturation	94°C	0.30	×35 Cycles	94°C	1.30	×45 Cycles
Annealing	50°C	0.30		47°C	1.30	
Extension	72°C	0.30		72°C	1.30	
Final extension	72°C	5	Single	72°C	7	Single
Hold	4°C	∞		4°C	∞	

Table 5: Histopathological grading of SCC.

Site of occurrence	Grade of tumor			Total
	Grade 1	Grade 2	Grade 3	
HC	19(86.36%)	03(13.64%)	00(0.00%)	22
OSCC	20(55.56%)	05(13.88%)	11(30.56%)	36
VSCC	06(100.00%)	00(0.00%)	00(0.00%)	06
Total	45(70.31%)	08(12.50%)	11(17.19%)	64



(A) WD-SCC with keratin pearls (H and E ,200×). (B) Transition from pseudostratified columnar epithelium to SCC (H and E, 200×). (C) Interconnected osseous trabeculae with loose fibrovascular stroma (H and E, 200×). (D) WD infiltrative neoplasm showing anisocytosis, anisokaryosis and keratin pearls (H and E, 100×).

Fig 1: Histopathology.

Gross pathology of SCC

Gross pathomorphology of affected parts were recorded (Supplementary Material 2). In HC, majority cases (n=12) exhibited cauliflower shaped growth at base of affected horn; while others showed nodular growth with verrucous surface (n=10). Most growths had a firm consistency. Some large ulcerated growth showed bleeding from wound. Cut sections revealed yellowish-white, grayish-white, reddish-brown to brownish-black surface along with widespread hemorrhage and necrosis (Desai *et al.*, 2024; Kumar *et al.*, 2025).

Majority OSCC (n=19) were found on nictitating membrane followed by lower eyelid, bulbar conjunctiva, corneo-scleral junction and upper eyelid, respectively. Majority had cauliflower like appearance followed by nodular growth with rough and verrucous surface. Few cases had papillomatous and pedunculated growth. Similar alterations were revealed by Fornazari *et al.* (2017), in contrast to Karakurt *et al.* (2023).

Histopathology of SCC

Histopathological grading was carried out and divided into WD, MD and PD tumors (Table 5). Scoring of individual tumorous mass was noted (Supplementary Material 3).

Supplementary Material 2: Gross pathomorphological features of suspected cases of SCCs.

Sample ID	Right/Left	Location of Neoplasm	Surface appearance (shape/ colour and consistency)
BoHC1	Right horn	Base of right horn	Round, nodular, firm ulcerative mass with rough and verrucous surface with purulent discharge
BoHC2	Left horn	Base of left horn, extending to frontal sinus and skull	Soft and friable, pink, large cauliflower like mass with broad base, hemorhhagic and foul smelling mass
BoHC3, BuHC2	Left horn	Base of left horn	Large, cauliflower like mass, turgid and hemorrhagic
BoHC4, BoHC11, BoHC16	Right horn	Base of right horn	Solid, nodular protruded out mass of tissue
BoHC5	Left horn	Base of left horn	Soft and friable, huge cauliflower like mass with broad base and severely hemorrhagic, maggot infestation
BoHC6, BoHC12, BoHC19	Left horn	Base of left horn	Small, hard, nodular mass having verrucous surface
BoHC7, BoHC18	Left horn	Base of right horn	Large, firm cauliflower like growth
BoHC8	Right horn	Base of right horn	Large, cauliflower like mass, turgid and hemorrhagic
BoHC9	Left horn	Base of left horn	Solid, nodular protruded out mass of tissue
BoHC10	Left horn	Base of left horn	Small, round nodular growth with severe foul smelling purulent discharge
BoHC13	Left horn	Base of left horn	Large, cauliflower like growth with firm consistency and maggot infestations
BoHC14	Right horn	Base of right horn	Small, hard, nodular mass having verrucous surface
BoHC15	Right horn	Base of right horn	Large cauliflower like growth, soft and friable mass of tissue
BoHC17	Left horn	Base of left horn	Soft, small irregular cauliflower like mass
BuHC1	Right horn	Base of right horn	Soft and friable, huge cauliflower like mass with broad base, crusty elevated growth, severely hemorrhagic
BuHC3	Right horn	Base of right horn, extending deep to frontal sinus	Round, nodular, hard ulcerative mass with foul smelling purulent discharge
BoEC1	Right eye	Nictitating membrane and lower eyelid of right eye	Round, nodular, firm ulcerative mass with rough and verrucous surface with purulent discharge
BoEC2	Right eye	Nictitating membrane and lower eyelid of right eye	Soft and friable, pink, large cauliflower like mass, hemorrhagic with foul smelling
BoEC3	Left eye	Bulbar conjunctiva and corneo-scleral junction	Large, cauliflower like mass, turgid and hemorrhagic
BoEC4	Left eye	Whole eyeball of left eye	Solid, nodular protruded out mass of tissue
BoEC5	Left eye	Whole eyeball of left eye	Soft and friable, huge cauliflower like mass with severe hemorrhage
BoEC6	Left eye	Haemorrhagic, small friable mass at lower eyelid of left eye	Small, hard, nodular mass having verrucous surface
BoEC7	Right eye	Bulbar conjunctiva and corneo-scleral junction	Large, cauliflower like growth with firm consistency
BoEC8	Right eye	Lower eyelid, limbus and nictitating membrane of right eye	Large, cauliflower like mass, turgid and hemorrhagic
BoEC9	Right eye	Bulbar conjunctiva, cornea and eyelid of right eye	Solid, nodular protruded out mass of tissue

Supplementary Material 2: Continue....

Supplementary Material 2: Continue....

BoEC10	Left eye	Bulbar conjunctiva, limbus and lower eyelid of left eye	Small, round nodular growth with severe foul smelling purulent discharge Soft, nodular protruded out mass of tissue
BoEC11	Right eye	Nictitating membrane of right eye	
BoEC12	Right eye	Lower eyelid of right eye	Small, hard, nodular mass having verrucous surface
BoEC13, BoEC18	Left eye	Whole eyeball of left eye	Large, firm cauliflower like growth
BoEC14	Right eye	Nictitating membrane and lower eyelid of right eye	Small, hard, nodular mass having verrucous surface
BoEC15	Right eye	Nictitating membrane of right eye	Large cauliflower like growth, soft and friable mass of tissue
BoEC16	Right eye	Nictitating membrane of right eye	Solid, nodular protruded out mass of tissue
BoEC17	Left eye	Lower eyelid of left eye	Soft, small irregular cauliflower like mass
BoEC19	Right eye	Nictitating membrane of right eye	Small, hard, nodular mass having verrucous surface
BoEC20	Right eye	Lower eyelid of right eye	Soft and friable, huge cauliflower like mass with severe hemorrhage
BoEC21	Left eye	Bulbar conjunctiva and corneo-scleral junction	Large, cauliflower like mass, turgid and hemorrhagic
BoEC22	Right eye	Nictitating membrane of right eye	Round, nodular, hard ulcerative mass with foul smelling purulent discharge
BoEC23	Right eye	Nictitating membrane of right eye	Cauliflower like appearance, ulcerated hard mass
BoEC24	Left eye	Bulbar conjunctiva and corneo-scleral junction	Papillomatous and pedunculated mass
BoEC25	Right eye	Lower eyelid of right eye	Cauliflower like with hyperkeratosis and ulceration
BoEC26, BoEC27	Right eye	Nictitating membrane of right eye	Large nodular, hemorrhagic growth with verrucous surface
BoEC28	Left eye	Bulbar conjunctiva and corneo-scleral junction	Large oedematous and turgid, cauliflower like mass, poorly demarcated with verrucous surface
BoEC29	Right eye	Nictitating membrane of right eye	Papillomatous and pedunculated mass
BoEC30	Right eye	Nictitating membrane of right eye	Cauliflower like appearance, crumbles on touching
BoEC31	Left eye	Upper eyelid	Large red irregular firm nodular mass, hemorrhagic and protruded out at bulbar conjuctiva
BoEC32	Right eye	Nictitating membrane of right eye	Hemorrhagic, small friable mass
BuEC1	Right eye	Nictitating membrane of right eye	Nodular hard mass
BuEC2	Left eye	Nictitating membrane of left eye	Cauliflower like appearance, crumbles on touching
BuEC3	Left eye	Nictitating membrane of left eye	Hemorrhagic, small friable cauliflower like mass
BuEC4	Left eye	Nictitating membrane of left eye	Cauliflower like growth
BoVC1	-	Ventral region of vulvar lips	Firm, dark red, nodular growth
BoVC2	-	Ventral region of vulvar lips	Blackish cylindrical, ulcerated nodular growth
BoVC3	-	Ventral region of vulvar lips	firm mass with severe purulent discharge
BoVC4	-	Ventral region of vulvar lips	large, red, cauliflower like growth protruding from vulva
BoVC5	-	Ventral region of vulvar lips	firm, ulcerated cauliflower like growth
BuVC1	-	Ventral region of vulvar lips	Ulcerated cauliflower shaped growth at distal end

Note: BoHC: Bovine horn cancer, BuHC: Bubaline horn cancer, BoEC: Bovine eye cancer, BuEC: Bubaline eye cancer, BoVC: Bovine vulvar cancer, BuVC: Bubaline vulvar cancer.

Histopathology of WD-HC revealed proliferating neoplastic cells forming concentric lamellae of keratins developing large epithelial pearls (Fig 1A). Prominent keratinization was observed in center of islands (Joshi *et al.*, 2009). Cells were arranged in islands and showed moderate pleomorphism. One case showed a transition between pseudostratified columnar epithelium to SCC (Fig 1B) and also showed interconnected osseous trabeculae that supported by loose fibrovascular stroma (Fig 1C). Mitotic index was very low. MD neoplasms revealed neoplastic cells arranged in cords and trabeculae and showed pleomorphism and apoptosis.

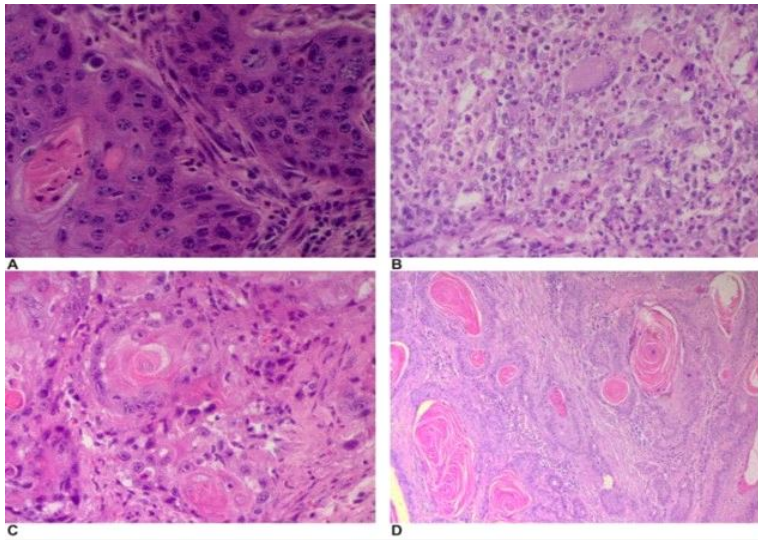
Well-differentiated OSCC revealed presence of round to polygonal shaped cells arranged in islands, cords and trabeculae, supported by fibrovascular stroma and typical keratin pearls (Fig 1D). Neoplastic cells had distinct cell borders and showed anisocytosis and anisokaryosis, karyomegaly, apoptosis and few mitotic figures (Fig 2A). Few cases revealed marked granulomatous inflammation characterized by infiltration of macrophages, multinucleated giant cells, plasma cells, lymphocytes and polymorphs cells (Fig 2B). WD and MD neoplasms showed multifocal neoplastic cell with prominent intercellular bridges (desmosomes) (Fig 2C). PD infiltrative neoplasm showed polygonal cells arranged in cords and trabeculae. Mononuclear cells infiltration and a typical mitotic figures were noted (Sharma *et al.*, 2020; Karakurt *et al.*, 2023).

VSCC showed proliferating neoplastic cells arranged in small islands that formed concentric layers, developing multiple prominent characteristic epithelial pearls (Fig 2D). Neoplastic cells showed minimal anisocytosis and anisokaryosis (Khodakaram-Tafti *et al.*, 2013).

Immuno histo chemistry of SCC

Immunohistochemical examination was performed using aforementioned various tumor markers. IHC revealed all cases of SCCs (100%) were positive for PCK, PCNA and p63; 42 (65.63%) for ZO-1; 35 (54.68%) for E-cadherin; 39 (60.94%) for p120; 52 (81.25%) for Vimentin; and 56 (87.50%) were for γ-H2AX. However, FOXA1 and GATA3 failed to react with any tumorous tissue. Immunohistochemical scoring of individual tumor mass for each marker is given in Supplementary Material 4.

Current study reported that all cases showed cytoplasmic immunoreactivity to PCK. Out of 64, 4(6.25%) cases depicted immunoreactivity in >75% neoplastic cells and were considered strongly immunoreactive (Fig 3A). However, 25(39.06%) cases showed expression in 51-75% cells, 24(37.50%) in 26-50% cells and 11(17.19%) in <25% cells. WD-SCC showed strong cytoplasmic immunoreactivity with pancytokeratin in neoplastic cells and keratin pearls (Fig 3B). The present findings were similar to those of Kumar *et al.* (2023). The strong immunolabelling of PCK indicates tumor of epithelial origin. Among 64 cases, 7 had nuclear immunoreactivity to PCNA in >75% of neoplastic cells and were considered strongly immunoreactive. However, 26 cases showed expression in 51-75% cells, 28 showed expression in 26-50% cells and 3 showed expression in 5-25% of cells. These findings suggested that WD-SCC showed mild to moderate nuclear immunostaining (Fig 3C), while PD-SCC showed strong



(A) Anisocytosis, anisokaryosis, karyomegaly, apoptosis and mitotic figures (H and E, 400×). (B) Granulomatous inflammation with macrophages, multinucleated giant cells, plasma cells, lymphocytes and polymorph cells (H and E, 400×). (C) WD-SCC showing prominent desmosomes (H and E, 400×). (D) WD-SCC with polygonal cells arranged in cords and trabeculae (H and E,100×).

Fig 2: Histopathology.

Supplementary Material 3: Histopathological scoring of individual SCC as per modified Anneroth's grading system.

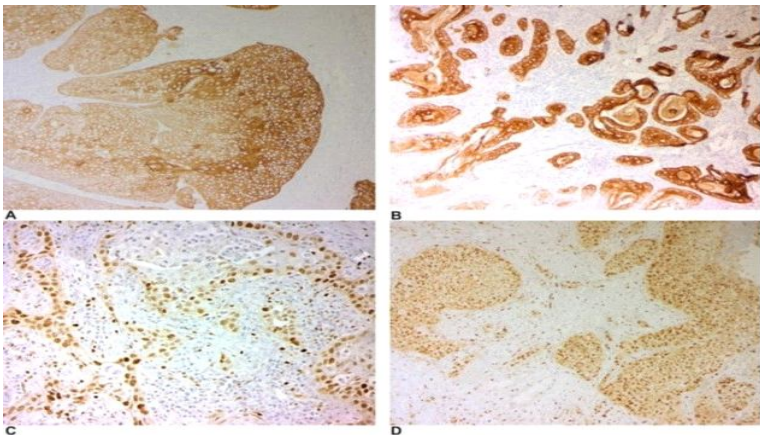
Sample ID	Degree of keratinization	Nuclear pleomorphism	Number of mitoses per HPF	Pattern of invasion	Total Score	Grade	Differentiation
BoHC1	1	1	1	2	5	I	Well differentiated
BoHC2	1	1	2	1	5	I	Well differentiated
BoHC3	1	1	1	3	6	I	Well differentiated
BoHC4	1	1	2	2	6	I	Well differentiated
BoHC5	1	2	1	1	5	I	Well differentiated
BoHC6	2	2	1	2	7	II	Moderately differentiated
BoHC7	1	2	2	3	8	I	Well differentiated
BoHC8	2	1	1	1	5	I	Well differentiated
BoHC9	1	1	1	2	5	I	Well differentiated
BoHC10	1	1	1	1	4	I	Well differentiated
BoHC11	1	2	2	1	6	I	Well differentiated
BoHC12	1	2	1	2	6	I	Well differentiated
BoHC13	1	1	1	1	4	I	Well differentiated
BoHC14	1	1	1	2	5	I	Well differentiated
BoHC15	2	2	1	2	7	II	Moderately differentiated
BoHC16	1	1	1	2	5	I	Well differentiated
BoHC17	1	2	1	2	6	I	Well differentiated
BoHC18	1	2	1	0	4	I	Well differentiated
BoHC19	1	0	1	1	3	I	Well differentiated
BuHC1	1	2	1	1	5	I	Well differentiated
BuHC2	1	1	1	2	5	I	Well differentiated
BuHC3	1	2	2	3	8	II	Moderately differentiated
BoEC1	3	3	2	2	10	III	Poorly differentiated
BoEC2	2	1	1	2	6	I	Well differentiated
BoEC3	1	2	1	2	6	I	Well differentiated
BoEC4	2	2	2	3	9	II	Moderately differentiated
BoEC5	3	2	2	3	10	III	Poorly differentiated
BoEC6	2	2	1	2	7	II	Moderately differentiated
BoEC7	3	2	2	3	10	III	Poorly differentiated
BoEC8	3	1	3	3	10	III	Poorly differentiated
BoEC9	3	3	2	3	11	III	Poorly differentiated
BoEC10	1	2	1	2	6	I	Well differentiated
BoEC11	1	1	1	2	5	I	Well differentiated
BoEC12	2	2	1	1	6	I	Well differentiated
BoEC13	1	2	2	1	6	I	Well differentiated
BoEC14	3	2	1	3	9	III	Poorly differentiated
BoEC15	2	2	1	1	6	I	Well differentiated
BoEC16	1	1	1	1	4	I	Well differentiated
BoEC17	1	2	1	1	5	I	Well differentiated
BoEC18	2	2	1	2	7	II	Moderately differentiated
BoEC19	2	1	1	1	5	I	Well differentiated
BoEC20	1	2	1	1	5	I	Well differentiated
BoEC21	1	1	1	2	5	I	Well differentiated
BoEC22	2	1	1	1	5	I	Well differentiated
BoEC23	1	1	1	1	4	I	Well differentiated
BoEC24	3	2	2	3	10	III	Poorly differentiated
BoEC25	2	2	2	2	8	II	Moderately differentiated
BoEC26	1	2	1	1	5	I	Well differentiated
BoEC27	1	1	1	1	4	I	Well differentiated

Supplementary Material 3: Continue...

Supplementary Material 3: continue...

BoEC28	1	1	1	1	4	I	Well differentiated
BoEC29	2	2	1	3	8	II	Moderately differentiated
BoEC30	3	2	2	3	10	III	Poorly differentiated
BoEC31	1	1	1	1	4	I	Well differentiated
BoEC32	1	2	1	1	5	I	Well differentiated
BuEC1	3	3	2	2	10	III	Poorly differentiated
BuEC2	1	1	2	1	5	I	Well differentiated
BuEC3	3	2	2	3	10	III	Poorly differentiated
BuEC4	3	3	3	3	12	III	Poorly differentiated
BoVC1	1	1	2	1	5	I	Well differentiated
BoVC2	1	2	1	1	5	I	Well differentiated
BoVC3	1	1	1	1	4	I	Well differentiated
BoVC4	1	2	1	2	6	I	Well differentiated
BoVC5	1	2	1	1	5	I	Well differentiated
BuVC1	1	1	1	1	4	I	Well differentiated

Note: BoHC: Bovine horn cancer, BuHC: Bubaline horn cancer, BoEC: Bovine eye cancer, BuEC: Bubaline eye cancer, BoVC: Bovine vulvar cancer, BuVC: Bubaline vulvar cancer, HPF: High power field.



(A) WD-SCC showing strong cytoplasmic PCK immunoreactivity in >75% neoplastic cells (100×). (B) Strong PCK immunoreactivity in keratin pearls (200×). (C) WD-SCC showing nuclear PCNA immunoreactivity (200×). (D) PD-SCC showing strong nuclear PCNA immunoreactivity (100×).

Fig 3: Immunohistochemistry.

immnunostaining in proliferating neoplastic cells (Fig 3D) that suggests proliferative potential and biological aggressiveness of the tumor. These findings were in accordance with Martano *et al.* (2016). However, Karakurt *et al.* (2021) did not find any significant difference.

Among 64 cases, only one case (1.56%) showed membranous immunostaining of ZO-1 in >75% neoplastic cells, 13(20.31%) in ≥50-<75% cells, 19(29.69%) in ≥25-<50 cells, 9(14.06%) in ≥10-<25 cells and 22(34.38%) were in <10% neoplastic cells. 54 cases showed weak immunostaining (Fig 4A), while 10 had moderate membranous staining (Fig 4B). All tumors exhibited low ZO-1 expression in current study (Qiu *et al.*, 2016; Upadhaya *et al.*, 2022), indicating disruption of tight junction integrity during malignant transformation.

Out of 64 SCCs, 35(54.68%) cases showed positive immunoreactivity with E-cadherin (Fig 4C) and 29 were found non-immunoreactive. Among the 35 positively expressed tumors, 20 cases (20/35; 57.14%) showed immunolabelling in <25% neoplastic cells and 15(42.86%) cases showed in 26-50% neoplastic cells. Upadhaya *et al.* (2022) reported complete disruption of the adherens molecule in all cases. This down-regulation of E-cadherin indicates disruption of adherens junctions in epithelial tissues as cells progresses towards malignancy.

Among 64 SCCs, 52(81.25%) cases were found immunopositive for vimentin (Fig 4D), while 12 cases were found non-immunoreactive. This suggests that EMT is crucial for tumor invasion and metastasis. The present finding was found compatible with Yu *et al.* (2015).

Supplementary Material 4: Immunohistochemical scoring of individual SCC.

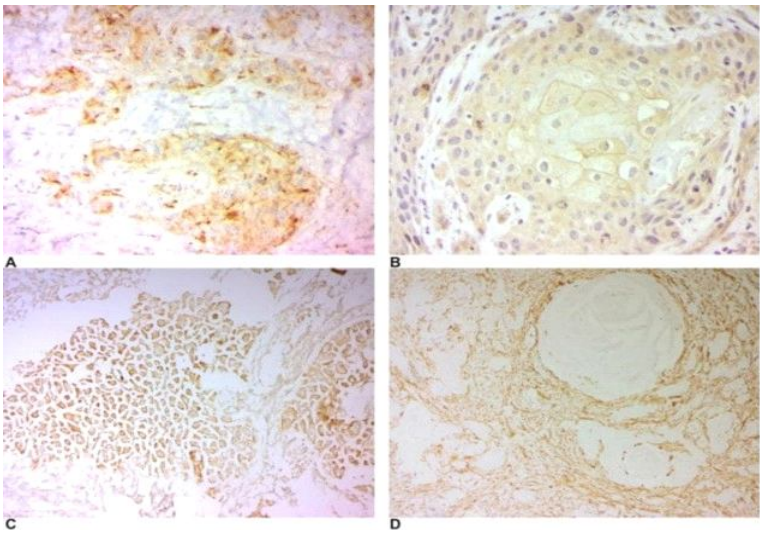
Case ID	PCK	PCNA	ZO-1			E-cad	Vim	p120	γ-H2AX	p63
			F	I	F×I					
BoHC1	4+	3+	4+	1	4	2+	N	3+	0	1+
BoHC2	3+	3+	2+	1	2	2+	N	3+	1+	1+
BoHC3	3+	3+	2+	2	4	1+	P	2+	1+	2+
BoHC4	2+	2+	0	1	0	2+	P	3+	2+	1+
BoHC5	3+	2+	1+	1	1	2+	P	2+	1+	1+
BoHC6	2+	3+	0	1	0	1+	P	0	2+	2+
BoHC7	3+	3+	3+	1	3	0	N	3+	0	2+
BoHC8	3+	3+	2+	2	4	2+	N	3+	0	1+
BoHC9	2+	2+	2+	1	2	1+	P	3+	2+	1+
BoHC10	3+	2+	3+	1	3	0	P	3+	1+	2+
BoHC11	2+	1+	1+	1	1	1+	P	1+	1+	1+
BoHC12	2+	2+	3+	1	3	2+	P	3+	1+	1+
BoHC13	3+	2+	2+	1	2	0	P	3+	2+	1+
BoHC14	3+	3+	2+	2	4	2+	N	3+	0	2+
BoHC15	1+	3+	1+	1	1	0	P	2+	2+	2+
BoHC16	3+	2+	3+	1	3	2+	P	3+	1+	2+
BoHC17	3+	3+	2+	1	2	1+	P	3+	1+	1+
BoHC18	4+	2+	3+	1	3	2+	P	3+	0	1+
BoHC19	4+	3+	2+	1	2	1+	N	3+	1+	1+
BuHC1	4+	2+	1+	2	2	1+	P	3+	0	2+
BuHC2	3+	2+	2+	1	2	2+	P	3+	2+	1+
BuHC3	2+	3+	1+	1	1	0	P	3+	3+	3+
BoEC1	1+	4+	0	1	0	1+	P	0	3+	3+
BoEC2	3+	2+	3+	1	3	0	P	3+	1+	1+
BoEC3	2+	2+	2+	1	2	1+	P	3+	1+	1+
BoEC4	3+	3+	0	1	0	0	P	1+	2+	2+
BoEC5	1+	3+	0	1	0	0	P	0	3+	3+
BoEC6	2+	4+	0	1	0	0	P	2+	2+	3+
BoEC7	1+	3+	0	1	0	0	P	1+	2+	3+
BoEC8	1+	4+	0	1	0	0	P	0	3+	3+
BoEC9	1+	4+	0	1	0	0	P	0	3+	3+
BoEC10	2+	2+	0	1	0	1+	P	3+	1+	1+
BoEC11	3+	2+	2+	1	2	0	N	3+	0	1+
BoEC12	3+	3+	2+	2	4	0	P	2+	1+	2+
BoEC13	2+	2+	0	1	0	0	P	1+	1+	1+
BoEC14	3+	3+	0	1	0	0	P	3+	3+	3+
BoEC15	3+	2+	2+	2	4	1+	P	3+	1+	2+
BoEC16	2+	2+	0	1	0	0	N	3+	0	1+
BoEC17	3+	3+	1+	1	1	0	P	3+	1+	2+
BoEC18	2+	2+	0	1	0	0	P	1+	2+	2+
BoEC19	3+	3+	3+	1	3	0	P	3+	1+	2+
BoEC20	2+	2+	2+	2	4	0	P	3+	1+	1+
BoEC21	2+	3+	3+	1	3	1+	N	3+	1+	2+
BoEC22	3+	3+	2+	1	2	0	P	2+	2+	2+
BoEC23	2+	2+	0	1	0	2+	P	3+	1+	1+
BoEC24	1+	4+	0	1	0	0	P	0	2+	3+
BoEC25	1+	3+	0	1	0	0	P	1+	2+	3+
BoEC26	3+	2+	2+	1	2	1+	P	2+	1+	1+

Supplementary Material 4: Continue...

Supplementary Material 4: Continue...

BoEC27	2+	3+	3+	1	3	2+	N	3+	1+	2+
BoEC28	2+	2+	2+	1	2	1+	P	3+	1+	2+
BoEC29	2+	3+	0	1	0	0	P	1+	2+	3+
BoEC30	1+	3+	0	1	0	0	P	1+	3+	3+
BoEC31	2+	2+	0	1	0	1+	P	3+	1+	2+
BoEC32	3+	2+	1+	2	2	1+	P	3+	1+	2+
BuEC1	1+	4+	1+	1	1	0	P	1+	3+	3+
BuEC2	2+	2+	3+	1	3	2+	P	3+	1+	2+
BuEC3	2+	3+	0	1	0	0	P	0	2+	3+
BuEC4	1+	4+	0	1	0	0	P	0	3+	3+
BoVC1	2+	2+	3+	1	3	1+	N	3+	1+	1+
BoVC2	3+	2+	2+	2	4	2+	P	3+	1+	1+
BoVC3	2+	3+	1+	2	2	1+	N	3+	2+	1+
BoVC4	3+	2+	3+	1	3	2+	P	2+	1+	2+
BoVC5	2+	1+	3+	1	3	1+	P	3+	1+	2+
BuVC1	3+	1+	2+	1	2	1+	P	3+	1+	2+

Note: BoHC: Bovine horn cancer, BuHC: Bubaline horn cancer, BoEC: Bovine eye cancer, BuEC: Bubaline eye cancer, BoVC: Bovine vulvar cancer, BuVC: Bubaline vulvar cancer, PCK: Pancytokeratin, PCNA: Proliferating cell nuclear antigen, ZO-1: Zonula occludence-1, E-cad: E-cadherin, Vim: Vimentin, γ-H2AX: Phospho-histone H2A.X, F: Frequency of positive cells, I: Staining intensity, P: Positive, N: Negative.



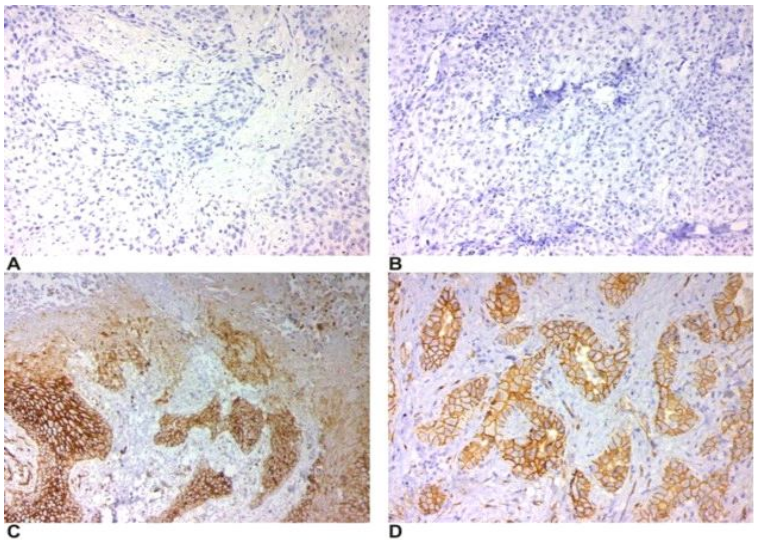
(A) PD-SCC showing decreased membranous ZO-1 expression (400×). (B) WD-SCC showing membranous ZO-1 immunoreactivity (400×). (C) SCC showing membranous E-cadherin immunoreactivity in individualized cells (200×). (D) WD-SCC showing vimentin immunoreactivity in fibrous connective tissue (200×).

Fig 4: Immunohistochemistry.

FOXA1 did not show any nuclear immunoreactivity in neoplastic cells and was thus considered as non-immunoreactive (Fig 5A). This negative immunoreactivity could be due to the antibody clone failing to react with bovine tissue. Similarly, all cases were found negative for GATA3 immunoreactivity (Fig 5B). Liu *et al.* (2012) also reported similar findings in SCC cases.

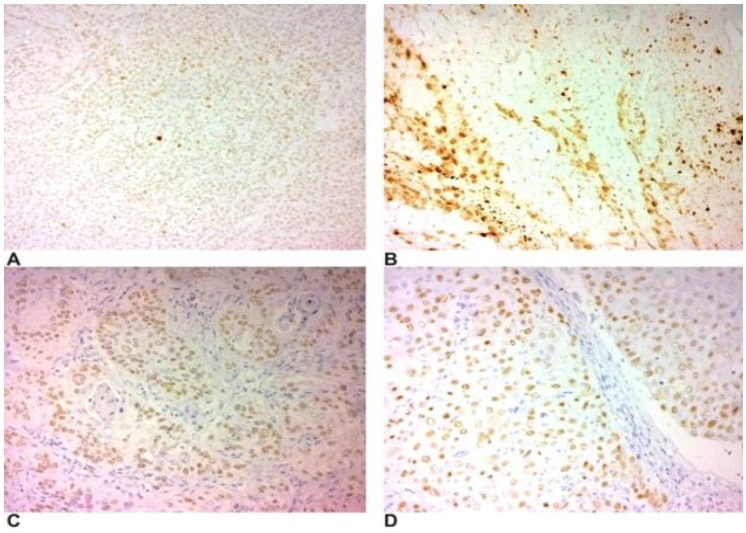
A total of 39(60.94%) cases showed p120 membranous expression in >50% neoplastic cells and were considered to show preserved expression of p120 (Fig 5C). Whereas, 25(39.06%) cases showed <50% of positive neoplastic cells and considered as reduced expression (Fig 5D). Li *et al.* (2020) also reported decreased expression in SCC**,** which may indicate loss of cell adhesion and enhanced invasive potential of neoplastic cells.

In this study, 56 (87.50%) were found immunoreactive for γ-H2AX whereas 8 (12.50%) were found negative. Out of 56, 31(55.36%) cases showed mild nuclear



MD-SCC showing absence of FOXA1 (200×) (A) and GATA3 (100×) (B) immunoreactivity. (C) WD-SCC showing preserved membranous p120 expression (100×). (D) PD-SCC showing reduced membranous p120 expression (200×).

Fig 5: Immunohistochemistry.



(A) WD-SCC showing mild γ-H2AX immunoreactivity (200×). (B) PD-SCC showing increased γ-H2AX immunoreactivity (200×). (C) WD-SCC showing p63 immunoreactivity restricted to the basal layers (200×). (D) PD-SCC showing diffuse p63 immunoreactivity (200×).

Fig 6: Immunohistochemistry.

immunoreactivity (Fig 6A), 16(28.57%) showed moderate immunoreactivity and 9(16.07%) showed severe immunoreactivity (Fig 6B). While 8(12.50%) cases were found non-immunoreactive similar to Dörtbudak and Yener (2025). Chronic ultraviolet (UV) radiation exposure is considered an important etiological factor that can induce DNA damage, leading to increased γ-H2AX expression.

Furthermore, all cases were found immunoreactive for p63 (Fig 6C) and (Fig 6D). A total of 15(23.44%) cases showed p63 expression in >70% neoplastic cells, 24(37.50%) cases in >50-<75% cells and 25(39.06%) cases in <25% neoplastic cells. In current study, p63 expression showed a significant correlation with the degree of tumor differentiation similar to Mestrinho *et al.* (2015).



Lane-1: 100 bp ladder, Lane-2 to 7: Positive samples (225 bp).

Fig 7: PCR amplification of mammalian IGF-1 gene.



Lane-1: 100 bp ladder, Lane-2 to 5: Positive samples (478 bp).

Fig 8: PCR amplification of *L*1 gene of *Papillomavirus*.

Molecular detection of oncogenic virus

All extracted DNA tested positive on the quality-assessment using PCR for *IGF-1* gene (Fig 7). Present investigation reported successful molecular detection of *Papillomavirus* in only four cases (6.25%) (Fig 8). Earlier studies were unable to detect BPV genome in SCC (Rutten *et al.*, 1992; Fornazari *et al.*, 2017). However, Karakurt *et al.* (2023) reported two positive cases in which one case revealed homology with BPV-1. Low detection rate could be attributed to the limited sensitivity of PCR primers used. Future studies employing broader BPV typing strategies may provide a more comprehensive assessment.

CONCLUSION

The present study demonstrated higher occurrence in cattle, particularly in crossbred Holstein-Friesian and aged female. Immunohistochemistry confirmed strong expression of PCK, PCNA, p120, p63 and γ-H2AX, highlighting their diagnostic and biological significance. PCR confirmed the presence of *Papillomavirus* in bovine SCC. However, future studies using broader BPV typing approaches are warranted.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

The research protocol was approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science and Animal Husbandry (COVSAH), KU, Sardarkrushinagar, Gujarat, vide letter no. VETCOLL/IAEC/2025/24/PROTOCOL-4.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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