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Pathological studies and detection of different bovine papilloma virus types in buffalo cutaneous warts

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Abstract: Bovine papillomatosis is a common viral disease of cattle but is relatively less common in buffaloes. Bovine papilloma virus (BPV) -1 and -2 are mostly associated with cutaneous warts in buffaloes. The aim of the present work was to study the pathomorphology and association of different BPV types in buffalo cutaneous warts. A total of 10 cases were included in the present study, which revealed that growths were either single or multiple, variable in shape and size, and present at different body sites. Histopathologically, these were categorized as papilloma, fibropapilloma, and fibroma. Polymerase chain reaction (PCR) revealed the presence of BPV-1, -2, and -5 DNA. Quantitative real-time PCR showed varying amounts of viral DNA copy numbers. On immunohistochemistry, nuclear Ki-67 expression was observed mainly in the cells of suprabasal layers. In conclusion, DNA of BPV-1, -2, and -5 was detected either alone or their mixed infections. Nuclear Ki-67 immunoreactivity in the suprabasal layer indicated that this is the cellular proliferation site.

Key words: Bovine papilloma virus, buffalo, cutaneous warts, Ki-67

Bovine papillomatosis (BP) is distributed worldwide among cattle but is relatively less common in buffaloes. Prevalence of this disease is established in buffaloes from India (1,2) and Italy (3) caused by bovine papilloma virus (BPV). BPV is a nonenveloped double stranded DNA virus with icosahedral symmetry that infects the cutaneous and mucosal epithelia inducing hyperplastic lesions. Viral capsid proteins production takes place only in productive infections. The virion particles assemble in the granular and cornified layers; mature virus particles are released on disintegration of dead squamous cells (4). So far, 13 different BPV types have been identified and characterized. These are classified as Deltapapillomavirus (BPV-1, -2, and -13), Epsilonpapillomavirus (BPV-5 and -8), and Xipapillomavirus (BPV-3, -4, -6, -9, -10, -11, and -12), with the exception of BPV-7, which belongs to an as yet unassigned papillomavirus genus (5–8). Papillomatosis in buffaloes was reviewed and it was opined that it is an almost unknown disease in buffaloes and is caused by BPV-1 and -2 or their mixed infections. Once infection of BPV establishes in buffaloes, it spread from buffalo to buffalo, with no intermediate involvement of cattle (2). Various markers are expressed during cell division, each with a specific function. Expression of Ki-67 nuclear antigen in all phases of the cell-cycle except for G_0 has been recently found to be the most reliable indicator of cellular proliferation (9). Keeping the above in mind, the present work was carried out with the objective to study the occurrence and association of different BPV types with cutaneous warts (CWs) in Indian buffaloes, and their pathomorphological assessment.

CWs/tumor biopsies from buffaloes were collected from the Referral Veterinary Polyclinic IVRI, Izatnagar, Government Veterinary Hospitals, different villages around Bareilly, India, and two archived samples from West Bengal, India. Biopsies were collected after administration of local anesthesia around the growth, samples were preserved in 10% buffered formalin for histopathological studies, and a part of samples showing gross lesions were also stored in sterile vials at –20 °C for molecular studies. Detailed gross observations with specific locations of growths/wart-like lesions were recorded. After proper fixation, the representative tissues were dehydrated in increasing grades of alcohol, cleared in xylene, and embedded in paraffin blocks. Sections with 4–5 µm thickness were cut and stained with hematoxylin and eosin (H&E) as per the conventional procedure.

DNA was extracted from the tissue samples using the Genomic DNA Mini Kit as per the manufacturer's

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instructions (Qiagen, Hilden, Germany). Specific primers targeting the L1 gene of BPV-1 and -2 were used for PCR amplification (10). Specific primers for BPV-5 and -10 were designed by targeting the L1 gene using molecular biology tools (DNASTAR software, Madison, WI, USA) from published sequences and commercially synthesized from Operon Biotechnologies, Genetix Biotec. The specific primers pairs for BPV-5 (forward 5'-actggctctaccaagctcaagg-3', reverse 5'-gacagaagggttaacggtctgca-3') and BPV-10 (forward 5'-tgcattcaataggcttgcagatgc-3', reverse 5'-cacctcgagaccacaaatgc-3') were used. The primers were expected to amplify the specific viral DNA template of sizes 301, 164, 266, and 422 bp for BPV-1, -2, -5, and -10, respectively. The thermal cycling conditions were composed of initial denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 52 °C (BPV-1 and -2), 62 °C (BPV-5), and 60 °C (BPV-10) for 40 s, with a 50 s extension at 72 °C and single cycle of final elongation of 10 min at 72 °C. The amplified DNA products were visualized by transillumination under UV light (Geldoc; Alpha Tech, San Leandro, CA, USA) in 1.5% agarose containing ethidium bromide (0.5 g/mL) as per standard procedures.

For quantification, the quantitative EvaGreen PCR assay was performed in Stratagene Mx-3000P real-time PCR cycler using commercial reagents as per the manufacturer's instructions. The same primer pairs were used for real-time PCR as for conventional PCR. The reaction set-up comprised 25 µL of EvaGreen PCR Master mix, 0.6 µL each of forward and reverse primer, 3.0 µL of DNA, and nuclease-free water to make a total of 50 µL reaction volume. The thermal profile for BPV-1 comprised three segments. Segment 1 consisted of initial denaturation at 95°C for 10 min followed by segment 2, which comprised 40 repetitive cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 25 s, and extension at 72 °C for 30 s each. In segment 3 dissociation curve analysis was performed. It comprised a single denaturation cycle at 95 °C for 1 min, partial renaturation to 65 °C for 30 s, and again denaturation at 95 °C for 30 s. The thermal profile for BPV-2 and -5 comprised three similar segments with annealing at 60 °C and 63 °C for 30 and 20 s, respectively. Then 10-fold serial dilutions of known positive purified PCR products of cattle cutaneous warts were made. The standard curve was obtained using dilutions from 10^{-3} to 10^{-8} and then viral DNA copy numbers were determined from the standard curve.

For immunohistochemistry, 4–5-µm-thick sections were taken on 3-aminopropyl-triethoxysilane coated slides (Sigma Chemicals). Sections were deparaffinized in xylene (2 changes of 5 min each) and then redehydrated using descending grades of alcohol. Thereafter antigen retrieval was carried out in a microwave oven by immersing the slides in citrate buffer (0.01 M citric buffer, pH 6.0) twice for 5 min each. Endogenous peroxidase quenching was done in 3% H_2O_2 in methanol for 30 min. Then the slides were rinsed thoroughly with phosphate buffer saline (PBS, pH 7.2). Nonspecific sites were blocked with 5% normal goat serum (Sigma Chemicals) in PBS for 30 min for Ki-67 expression and washed again with PBS (three times for 5 min each). Sections were then incubated overnight at 4 °C with primary antibody (mouse monoclonal anti-Ki67, 1:50, mAb PP-67, P6834, Sigma Chemicals, USA) in PBS containing 1% bovine serum albumin (BSA). The negative controls were covered with diluent only. After washing with PBS, the sections were incubated with biotinylated secondary antibody and peroxidase as per the manufacturer's instructions (Sigma). Using 3-amino-9-ethyl-carbazole (AEC; Sigma Chemicals) as staining substrate, sections were stained for 3–10 min and then counterstained lightly for 3–5 min with Mayer's hematoxylin (MHS-16; Sigma). Finally, the slides were rinsed for 5 min in running tap water and mounted in glycerol. The fraction of immunopositive cells was counted as described previously (11). Firstly, the entire section was screened to find the region with the maximum number of positively stained nuclei in one microscopic field (magnification 40× objectives). The fraction of positive stained nuclei in this region was Ki-67_{max} (Ki-67_{max} = Number of nuclei positive for Ki-67 divided by the total number of nuclei in this selected field) and Ki-67_{tot} (Ki-67_{tot} = Number of nuclei positive for Ki-67 divided by the total number of nuclei in all the fields used for counting). The estimation of Ki-67_{tot} was based on the assessment of positivity in 5–10 consecutive microscopic fields. The Ki-67 labelling index was calculated by counting at least 1000 tumor cells and expressed as a percentage.

It has been established that both BPV-1 and -2 are the etiological agents of cutaneous and teat warts in cattle (12,13). BP is a known disease in cattle, while in buffaloes few sporadic cases have been reported in the Indian subcontinent and it is an unrecognized entity (2). In the present study, a total of ten cases of CWs in buffaloes were studied. The details of gross observations, histopathological diagnosis, and detection of BPV types in the CWs in buffaloes are presented in the Table. Grossly, these growths were either single or multiple and located at different parts of the body, i.e. near base of horn, shoulder region (Figure 1a), tip of ear pinnae (Figure 1b), leg, and ventral surface of tail near its base. Histopathologically, fibropapilloma (exophytic type) was the most common type while others were fibropapilloma (endophytic type), papilloma, and fibroma with a tendency towards fibrosarcoma. Microscopically, papillomas were characterized by well-developed finger-like projecting papillae with overlying

Table. Gross observations, histopathological diagnosis, and detection of BPV types in cutaneous warts in buffaloes.

S. Samples No.	Age (years)	Place of collection	Gross observations	Histopathological diagnosis	PCR results			
					BPV-1	BPV-2	BPV-5	BPV-10
1. BW-1	2.5	Sardar Nagar, Bareilly	Dome shaped wart-like lesion (approx. 1 cm in diameter) on left scapular region	Fibropapilloma	+	-	-	-
2. BW-2	3	Sardar Nagar, Bareilly	A small growth at the tip of left ear pinna	Fibropapilloma	-	-	-	-
3. BW-3	3	Kohmi Pratapur, Bareilly	A small wart-like projected growth below knee joint on left foreleg	Fibropapilloma	+	-	-	-
4. BW-4	3.5	Rehpura, Bareilly	A small flat growth on left hind leg on rear side	Papilloma	-	-	-	-
5. BW-5	4.5	Bhuta, Bareilly	A small projected outgrowth on the right hind limb	Papilloma	-	-	-	-
6. BW-6	6	Referral Veterinary Polyclinic, IVRI, Izatnagar	Dome shaped (approx. 2 cm Izatnagar diameter) outgrowth on right foreleg at the level of knee joint	Fibrosarcoma	+	-	-	-
7. BW-7	1	Ahladpur, Bareilly	A small round outgrowth on the dorsal surface of tail above switch	NP	-	-	-	-
8. BW-8	4	Referral Veterinary Polyclinic, IVRI, Izatnagar	Very small pin-head sized growths on the back and rear part of body	NP	+	-	+	-
9. BW-9	6	West Bengal	Cutaneous warts on body	Fibropapilloma	+	-	-	-
10. BW-10	5	West Bengal	Cutaneous warts on body	Fibropapilloma	+	+	-	-

NP: Not processed

stratum corneum, acanthosis, and uniformly down growing rete pegs (Figure 1c). Moderate degree of ortho or parakeratotic hyperkeratosis and negligible fibrocellular reaction were observed. Fibropapillomas revealed moderate to extensive hyperkeratosis, acanthosis, and koilocytes with eosinophilic and vacuolated cytoplasm, and condensed hyperchromatic, centrally, eccentrically placed or crescent-shaped nuclei. The granular cell layer revealed prominent, basophilic keratohyaline granules in cytoplasm (Figure 1d). Well-developed finger-like projections with connective tissue cores and proliferation of dermal fibrous connective tissues were noted. One case revealed proliferation of fibrous connective tissue with variable sized fibrocytes and fibroblasts containing pleomorphic and hyperchromatic nuclei. Few mitotic figures were also noted. In tumor stroma mixed cells infiltration (neutrophils and lymphocytes) was observed. This was diagnosed as fibroma with a tendency towards fibrosarcoma. Similar types of CWs were reported previously (1,3,14,15). In general, the warts were single and not widespread throughout the body as compared to cattle reported from various parts of the world. Mostly, the warts observed in the present study were located at the common sites of scratching. The papilloma virus tends to manifest lesions at the site of trauma and probably the reactivation of its latency leads to tumor development (16,17).

Out of 10 cases, BPV-1 was detected in 4 and 1 case each revealed mixed infections of BPV-1 and -2 and BPV-1 and -5 by PCR (Figure 2). BPV-1 and -2 have been reported previously in buffoloes (1,3,15); however, to the best of the authors' knowledge, it was for the first time BPV-5 DNA was detected in buffalo CWs in India. However, BPV-5 has

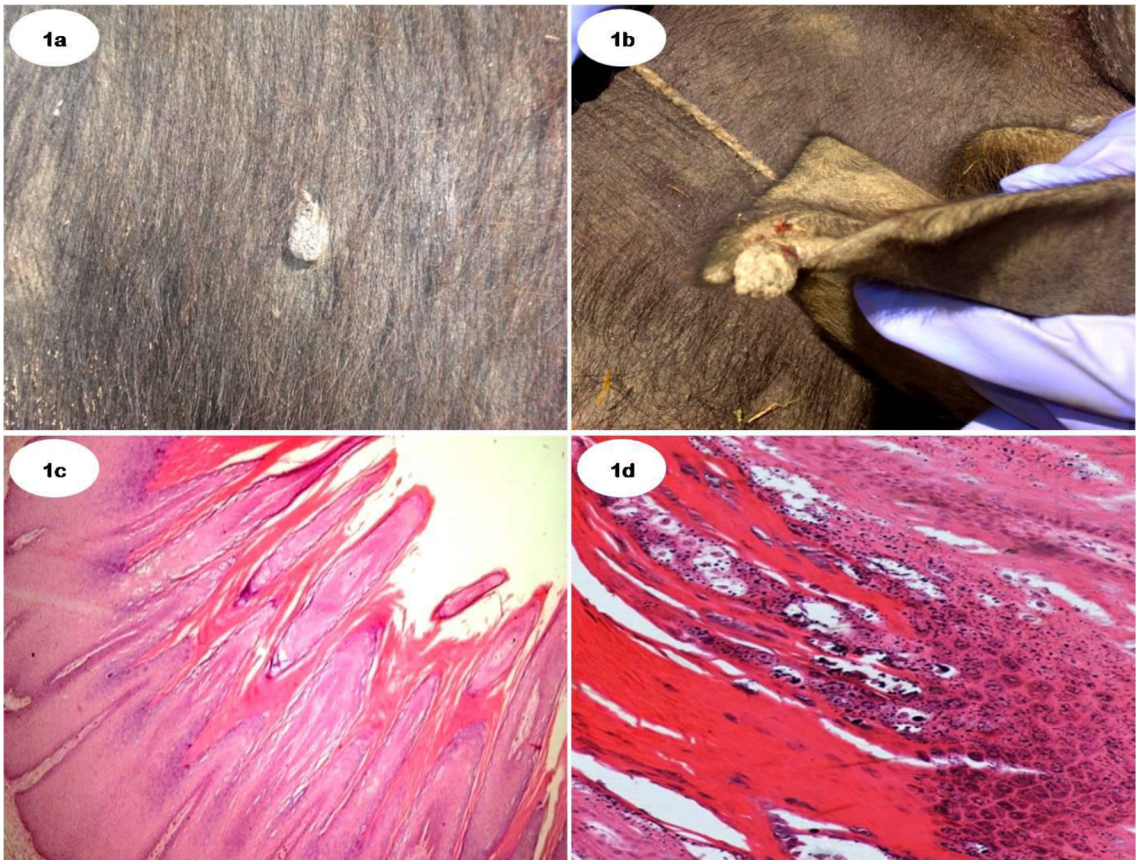

Figure 1. a: Small, dome shaped wart-like growth on shoulder region; b: Solitary, grayish, protruding growth on pinnae of the ear; c: Well-developed finger-like projecting papillae with overlying stratum corneum, acanthosis, and uniformly down growing rete pegs with no dermal fibrous hyperplasia. Papilloma, H&E ×40; d: Parakeratosis and marked hyperplasia of stratum granulosum with prominent basophilic keratohyaline granules. Fibropapilloma, H&E ×400.

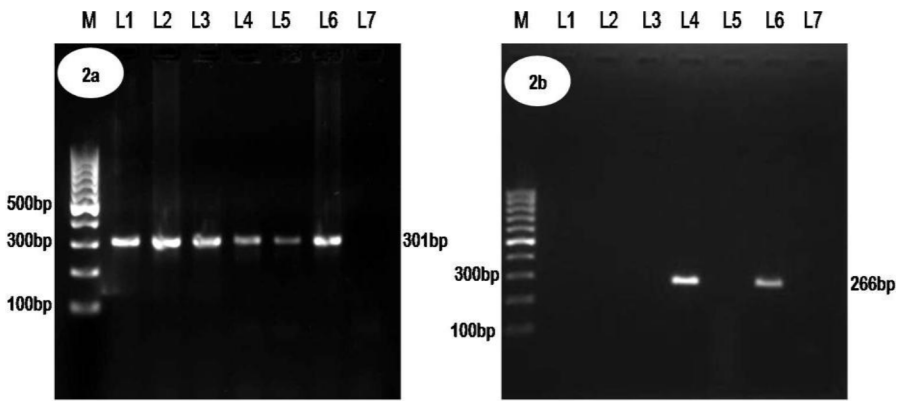

Figure 2. a: PCR products in ethidium bromide stained 1.5% agarose TBE gel electrophoresis. M-100 bp marker; L1–L5 (buffalo cutaneous warts) and L6 (positive control, cattle cutaneous warts): BPV-1, 301 bp; L7: no template control; b: PCR products in ethidium bromide stained 1.5% agarose TBE gel electrophoresis. M-100 bp marker; L4 (buffalo cutaneous wart) and L6 (positive control, cattle cutaneous warts): BPV-5, 266 bp; L7: no template control.

been detected either alone or mixed infection with BPV-1/-2 in mucosal warts in buffaloes (18). Mixed infections of different BPV types have been reported in cattle warts from different parts of world (15,19). In the present study, detection of different BPV types either alone or mixed infections supports the transmission of BPV from cattle

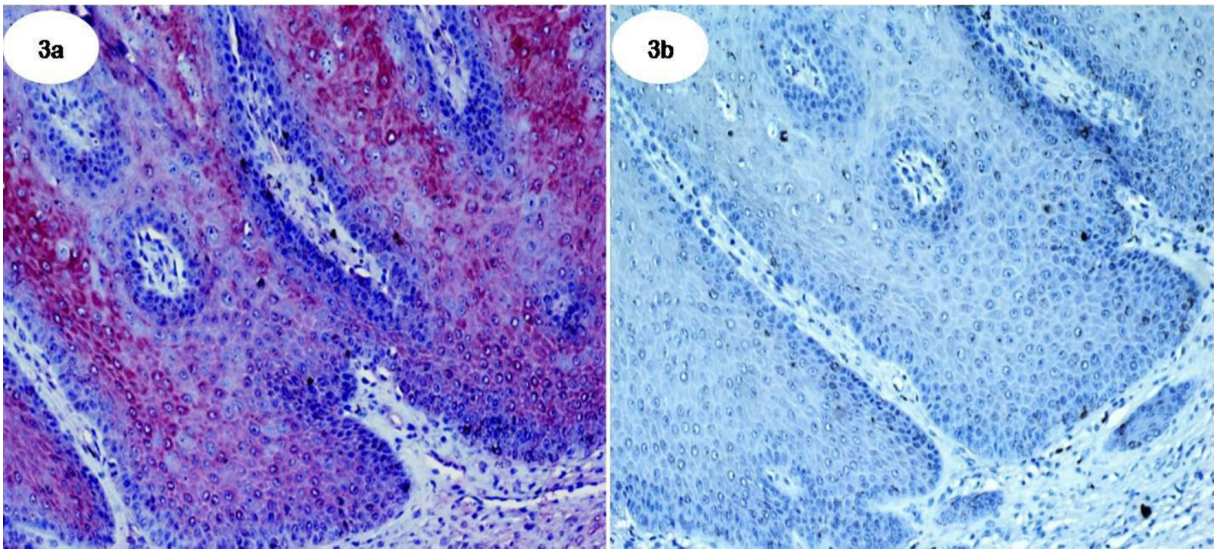

Figure 3. a: Immunohistochemical expression of Ki-67 mainly in stratum spinosum and mild in stratum granulosum with intense reddish intranuclear staining. Fibropapilloma, Ki-67, IHC, AEC ×400; b: Negative control showing no immunostaining for Ki-67. Fibropapilloma, Ki-67, IHC, AEC ×400.

to buffaloes as these are kept together in the same herd or share a common pasture. Quantitative real-time PCR revealed copy numbers of BPV-1, -2, and -5 ranging from 1.672e+003 to 1.281e+006, 3.974e+005, and 2.981e+005, respectively. The copy numbers in the buffalo CWs were much lower compared to cattle warts as reported earlier (15). The low copy number of viruses in the present study shows that the severity of papillomatosis was less as compared to cattle.

A total of eight buffalo CW samples were subjected to immunostaining for expression of Ki-67. Only two cases of fibropapilloma, BW-1 ($Ki\text{-}67_{max}$, 16.33%; $Ki\text{-}67_{tot}$, 11.89%) and BW-10 ($Ki\text{-}67_{max}$, 16.78%; $Ki\text{-}67_{tot}$, 13.23%), and one case of fibroma with tendency to fibrosarcoma, BW-6 ($Ki\text{-}67_{max}$, 27.67%; $Ki\text{-}67_{tot}$, 21.39%), showed immunostaining. It was restricted mainly to the cells of the suprabasal layers except for a few cells in the basal layer. Predominantly, nuclear staining was observed, but occasionally cytoplasmic and membranous staining was also observed in the stratum granulosum layer (Figure 3a). Negative controls did not show Ki-67 immunoexpression (Figure 3b). The pattern of Ki-67 expression in buffalo CWs was restricted to the suprabasal layer mainly in the stratum spinosum and upper layers except for a few cells in the basal layer as reported earlier (9,15). Similarly, in cattle warts cases, the intensity of immunostaining was less in buffaloes as reported previously (15). It is reported that Ki-67 expression is required for human papilloma virus replication (20). The present investigation also revealed similar expression behavior of Ki-67 in buffalo warts; However to interpret the result in relation with BPV further detailed studies on larger numbers of samples are needed. Ki-67 immunopositivity mainly in the suprabasal and occasionally in basal layers indicated these as cellular proliferation sites.

In conclusion, the severity of infection is less in comparison to it may be stated that cattle herds. BPVs were detected in the buffalo wart cases, but more elaborative and extensive studies are needed to elucidate the role of other BPV types in such infections as in cattle.

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