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BPV–2 associated papillomatosis in Indian water buffaloes

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ABSTRACT

Twenty cases of cutaneous warts (CWs) were recorded. Grossly and microscopically these were similar to those described in cattle as cauliflower-like or dome shaped and diagnosed as fibropapilloma/papilloma. These were characterized by presence of koilocytes, keratohyaline granules and inclusion bodies. BPV-like particles were demonstrated by negative staining and transmission electron microscopy (TEM). BPV–2 was detected from CWs by PCR and confirmed by nucleotide sequencing and phylogenetic analysis. CWs were successfully transmitted to hamsters, cattle and buffaloes. Lesions produced in hamsters were early fibromatosis to fibromas and those in cattle and buffaloes were identical to those in natural cases. This is the first confirmed report of BPV–2 associated papillomatosis in Indian water buffaloes and its transmission.

Key words: BPV–2, Buffalo papillomatosis, Fibropapilloma, Koilocytes

Cutaneous papillomatosis (warts) in bovine is a contagious hyperplasia or benign neoplasm. Bovine papilloma virus (BPV) have specific tropism for squamous epithelial cells and full viral replication, including synthesis of DNA, capsid proteins and assembly of virions, occur only in the more terminally differentiated squamous epithelial cells (Campo 2006). There are 10 well characterized types of BPV-l to -10, producing papilloma and fibropapilloma (Shinichi *et al*. 2008). BPV-8 was detected in Europian bison, and BPV–7, 9 and 10 were isolated from cattle in Japan (Ogawa *et al*. 2004,2007, Hatama *et al*. 2008). The aim of present study was to establish prevalence of CWs in Indian water buffaloes as a separate ailment and characterize its etiopathology in natural and experimental hosts.

MATERIALS AND METHODS

Spontaneous case studies

Field survey and sample collection: Survey was conducted in 6 organized dairy farms, local private dairies, large animal slaughter house, animal shows, etc. in Uttar Pradesh and Uttarakhand, India, to record the incidence of CWs/cases in buffaloes. The size, numbers, location of warts as well as age of animals and duration of affection were recorded. CWs tissue biopsies (12) and 5 blood samples were collected along with skin and blood samples from apparently normal buffaloes.

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DNA analysis

DNA was extracted from skin (one), lymphocytes (five) and CWs (ten) samples using DNA mini kit. Primers for conserved region of LI gene were used as BPV-1 (Fw: 5'-gga gcg cct gct aac tat agg a–3'; Rev: 5'-atc tgt tgt ttg ggt ggt gac–3'), BPV–2 (Fw: 5'-gtt ata cca ccc aaa gaa gac cct–3'; Rev; 5'-ctg gtt gca aca gct ctc ttt ctc–3' (Leishangthem 2008a). PCR products were electrophoresed on 1.5% agarose gel and visualized by transillumination under UV light.

Phylogenetic analysis

The PCR products were got directly sequenced commercially using primers and the sequences were compared with published sequences (EMBL and NCBI) of BPVs by phylogenetic analysis using EDITSEQ and MEG ALIGN modules of LASERGENE software.

Negative staining and TEM

BPV was purified from CWs as described by Lancaster and Olson (1978) and 1 mm^2 pieces were preserved in chilled 2.5% gluteraldehyde in 0.2 M phosphate buffer (pH 7.4) and stored at 4°C. Samples were processed and examined under electron microscope.

Pathological studies

CWs were biopsied and collected in 10% buffered formalin for routine histopathology and histochemistry (AgNOR). One sample of malignant urinary bladder tumor

(transitional cell adenocarcinoma) was examined in order to compare the degree of malignancy (Crocker 1992).

Apoptotic studies

Single cell suspensions of CWs prepared as described earlier (Nicolleti *et al*. 1991) were used for flow cytometric analysis using fluorescence activated cell sorter (FACS) calibur at Division of Biochemistry of the Institute. These were also examined for fluorescent pattern using fluorescent dyes acridine orange-ethidium bromide and Hoechst under fluorescent microscope and percentage of apoptotic cells were calculated.

Apoptotic index = (total number of apoptotic cells/total number of cells counted)×100.

Experimental transmission studies

The studies were done in both natural as well as experimental hosts.

Hamsters: Seven adult male Syrian golden hamsters (*Mesocricetus auratus*), weighing about 110–130 g in were used as infection and control groups containing 4 and 3 animals, respectively. They were infected by multiple scarification and inoculation (3 times) on the abdominal skin with homogenized suspension of CWs. All animals were observed regularly and sacrificed at 120 days post inoculation (DPI) and tissues were collected for routine histopathology and PCR analysis.

Cattle and buffaloes: A group of 6 adult animals consisting of 3 cattle in group 1 and 3 buffaloes in group 2 were experimentally infected by multiple scarifications and subcutaneous inoculation on neck, twice a week, with 1.0 ml homogenized suspension of CWs, tested positive for BPV–2. They were observed regularly for growth patterns on the site of inoculation and biopsies were collected in 10% buffered formalin on 70 DPI and processed for histopathology.

Both small and large animal experimentation were cleared by Institute Animal Ethics Committee. All the hamsters were sacrificed humanely under chloroform anaesthesia and skin biopsies from large animals were collected under local anaesthesia by skilled veterinarians.

RESULTS AND DISCUSSION

Clinical observations

In the present study, 19 cases of CWs (Fig.1) and 1 case of teat papilloma were recorded in buffaloes. Grossly, warts were cauliflower-like with horny papillae or dome shaped with smooth outer surface (0.5–2.0 cm in diameter) varying in number from 1 to 6. Commonly affected sites were head, face, neck, legs and back. Most of the cases (14/20) were recorded in adult milking Murrah buffaloes with only few cases (6/20) noticed in young animals. Bovine papillomatosis in cattle is a known disease while, in buffaloes few sporadic cases have been reported that too in the Indian subcontinent only and it is unrecognized entity (Joshi *et al*. 1994, Sood *et al*. 2006, Leishangthem *et al.* 2008a).

Etiological studies

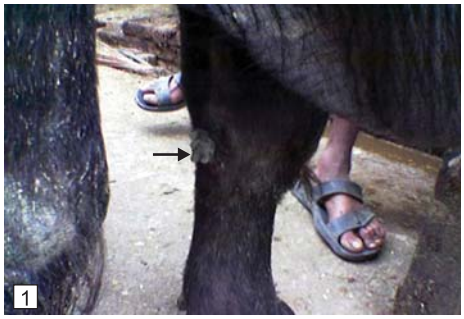
PCR: BPV–2 was detected in CWs (Fig.2) and lymphocytes of CWs affected animals. On phylogenetic analysis of the sequence of PCR products, 98% and 100% homology was seen with the genome sequences (Ac. No: M 20219 and XO 1768) and (Ac. No: EF 151531 and EF 151532) of BPV–2, respectively. Presence of BPV DNA in the lymphocytes supports its latency in the lymphocytes. Earlier, BPV was detected from CWs, blood and various tissue fluids of BP affected animals (Freitas *et al*. 2003, Leishangthem *et al*. 2008b).

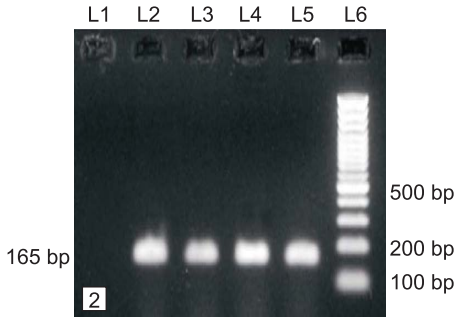
Negative staining and TEM: Negative staining of purified virus suspensions of CWs showed PVs-like particles of approximately 50–55 nm in size against dark background (Fig.3). Earlier Leishangthem *et al.* (2008a) also observed scanty virus particles by negative staining of cattle CWs. There were marked changes in the epidermocytes with high nucleus to cytoplasmic (N/C) ratio, pleomorphic and bizarre shaped nuclei, prominent heterochromatin, electron dense cytokeratin filaments, indistinct cell membrane and marked intercellular spaces. Cytoplasm, had multiple, small vacuoles with pyknotic nucleus and small aggregate of electron dense PVs-like particles of about 50–55 nm in size were seen (Fig 4–5). These finding are in accordance with those described for papillomatosis in cattle by earlier workers (Dorte *et al*. 2004, Joshi *et al*. 1994).

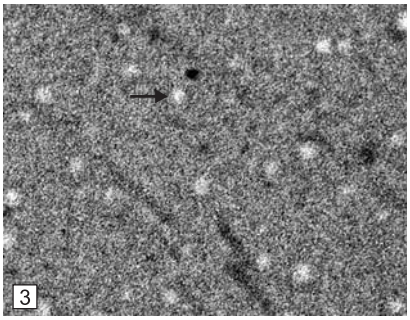
Pathological studies

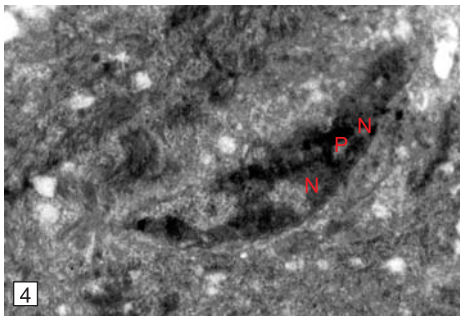
Gross and histopathology: A total of 12 cases of CWs were studied in detail and 2 types of growth patterns were seen. Grossly, 8 cases were of cauliflower-like with papillary outgrowths or dome shaped with smooth or undulating outer layer. Histologically, moderate to extensive cornification (hyperkeratosis) was seen with varying degree of parakeratosis (nuclear remnants), finger-like outgrowths (papillae) and dermal fibrous connective tissue core. Granular cell layer had prominent basophilic keratohyaline granules. Moderate to severe degree of acanthosis (thickening of stratum spinosum) was observed with irregular rete pegs projecting deep into the dermis with lateral inter-connections forming islands of dermal connective tissue surrounded by hyperplastic epidermal cells. Stratum spinosum revealed koilocytes, degenerating cells and intracytoplasmic eosinophilic inclusion bodies. Basal cell layer was hyperplastic with few mitoses. In hypodermis there were engorged capillaries with occasional infiltration of mononuclear cells and neutrophils. Extensive fibrocellular proliferative changes were seen in the hypodermis. These cases were diagnosed as fibropapilloma (exophytic, cauliflower-like or dome shaped).

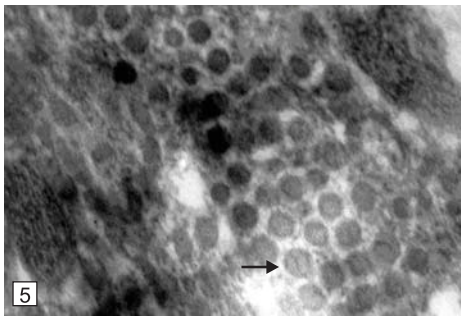
Four cases were cauliflower-like with papillary

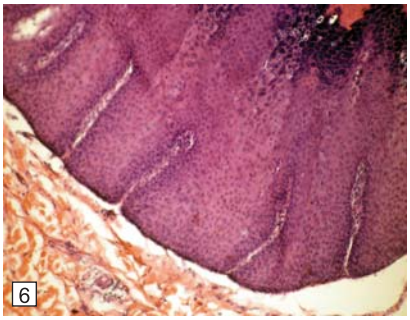


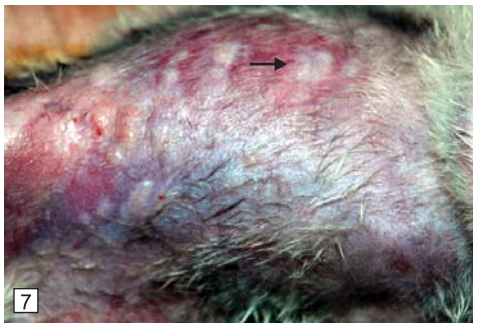


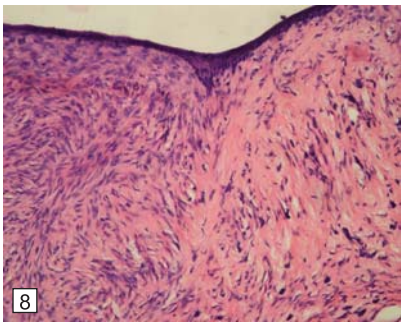


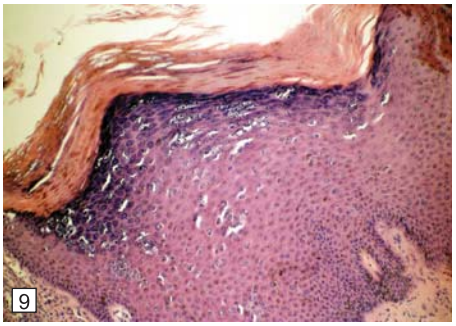


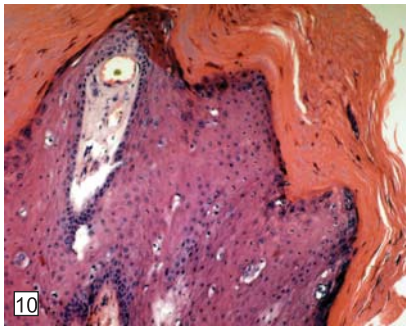












Figs 1–10. **1.** Cauliflower (arrow) like growth on fore leg. **2.** Dairy, IVRI. Buffalo: L1-negative control, L2–L5 CWs, M-100bp marker. **3.** Purified virus: virus-like particles (arrow) in buffalo Cutaneous Warts negative stain, EM×1,50,000. **4.** Epidermocyte with pyknotic nucleus (pN) and crystalline array of virus particles in nucleus (N), EM×4400. **5.** Papilloma virus-like particles (arrow) of 50–55 nm (scale not shown) in size. EM×3, 00,000. **6.** Acanthosis, uniformly downward growing columns of rete peges with no dermal fibrous hyperplasia. HE×100. **7.** Buffalo CWs infection: Multiple (10–12), small lentil sized tumours (arrow) on abdominal skin of hamster. **8.** Hamster dermal tumour: Areas with extensive connective tissue proliferation and well formed fibromas. HE×100. **9.** Buffalo infected with BCWs: Hyperkeratosis, acanthosis and proliferative changes in the dermal fibrous connective tissue, Fibropapilloma. HE×100. **10.** Cattle infected with BCWs: Finger-like papillae, hyperkeratosis, acanthosis, fibro-proliferative changes in the dermis. Fibropapilloma. HE×100.

outgrowths. Histopathologically, extensive hyperkeratosis and moderate to severe parakeratosis with basket wave appearance were seen in the stratum cornium. There were extensive acanthosis and uniformly downward growing columns of epidermal cells with thin core of dermal tissue (Fig.6). Whole epidermal layer was hyperplastic with hyperchromatic nuclei and few mitoses in basal cell layer. Stratum granulosa had intensively stained basophilic keratohyaline granules, which were seen with few koilocytes, and intracytoplasmic eosinophilic inclusion bodies were noticed in the upper layers. There were no fibrocellular proliferative changes in the hypodermis. These cases were diagnosed as papilloma (endophytic, cauliflower-like).

Earlier, this type of histological classification was not attempted in BP. However, fibropapilloma in cattle with acanthosis were described by Lancaster and Olson (1982) with koiocytes, keratohyaline granules and inclusion bodies (Jelinek and Tachezy 2005 and Tomita *et al*. 2007).

Histochemistry (*AgNORs count*)

The AgNOR dots were in general large and round in CWs and the average count were 1.9±0.43 whereas it was 3.9±0.34

in malignant transitional cell adenocarcinoma, indicating relative benign nature of CWs.

Apoptosis studies

FACS revealed high values of apoptotic cells (5 to 35%) indicating large aggregates of dead keratinocytes and confirm earlier findings (Leishangthem *et al.* 2008a). With fluorescent dye, apoptotic index of 12–20 (AO/EB) and 8–15 (HO) was recorded in CWs while it was 3–5 for normal skin. Apoptotic live and dead cells could be differentiated with fluorescent staining, and this is in accordance with earlier report that PVs sensitizes cells to apoptosis through DNA fragmentation (Dorte *et al*. 2004).

Experimental transmission studies

Hamsters: Grossly, few to multiple (3–12) small, lentils to pea-sized, nodular growths were noticed on the abdomen region in all the 4 animals in the infected group (Fig.7). The epithelium was intact with moderate to extensive proliferative changes in the dermal fibrous connective tissue. Fibroblasts and fibrocytes were seen with homogenous glossy mass of eosinophilic collagen fibers in radiating or whirling pattern (Fig.8). There was no encapsulation and mass was merged with the surrounding connective tissue and muscle. No mitoses were observed in the fibroblasts. Hyperplasia of sebaceous glands was seen with some epithelial proliferation around hair follicles. These cases were diagnosed as early fibromatosis to fibromas. The findings are similar to the earlier reports of progressing fibromas, fibromas and fibroblastic tumors induced by inoculation of cattle CWs (Lancaster and Olson 1982, Somvanshi *et al*. 1988).

Cattle and buffaloes: Grossly, small millet to pea nut sized warts-like growths were seen along the line of scarifications on 60–70 DPI in both groups, in 2 cows and 2 buffaloes. In buffaloes, the lesions were similar to those in natural cases, both grossly and microscopically and were diagnosed as cauliflower-like exophytic fibropapilloma (Fig.9) and endophytic papilloma in buffaloes while fibropapilloma (exophytic, dome shaped) in cattle (Fig.10). It indicated that buffalo CWs are transmissible to both cattle as well as buffaloes. Experimental transmission of cattle CWs was reported in calves (Meischke 1979). Recently, CWs in a cattle and European bison in Japan were not found identical to any of the BPVs and were established as new types.

To sum up, this is the first systematic study of spontaneous cases of CWs in Indian water buffaloes with an aim to establish as new disease entity. BPV-like particles were demonstrated by negative staining and TEM of CWs in buffaloes. CWs of buffaloes are associated with BPV–2, are transmissible to hamster as well as cattle and buffaloes and induce hyperproliferation of keratinocytes and fibroblasts. The lesions produced in hamsters were fibromatosis to fibroma whereas in cattle and buffaloes fibropapilloma along with endophytic papilloma which have not been described earlier. This is only second example of interspecies transmission of papilloma viruses in any species, first being equine sarcoids due to BPV-1 and –2. However, complete genome sequencing of viral DNA from buffalo CWs and its comparison with that of BPV–2 needs to be done to know per cent of homology between the two. Also, cases of endophytic papilloma, observed in both spontaneous and experimental cases of CWs in buffalo needs to be investigated further, as it is not common in cattle.

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