DETECTION OF BPV-2 IN CUTANEOUS WARTS OF INDIAN WATER BUFFALOES (Bubalus bubalis)


ABSTRACT

A study was undertaken to investigate the incidence and establish etiopathology of cutaneous warts (CWs) in Indian water buffaloes of the Murrah breed in parts of Uttar Pradesh district of India. Twenty cases of CWs were recorded. Grossly and microscopically they were cauliflower-like or dome-shaped and were diagnosed as fibropapilloma/papilloma. They were characterized by presence of koilocytes, keratohyaline granules and inclusion bodies. Negative staining and transmission electron microscopy (TEM) revealed bovine papilloma virus (BPV) like particles. BPV-2 was detected from CWs by PCR and was confirmed by nucleotide sequencing and phylogenetic analysis. Further, 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 in buffaloes. This is the first confirmed report about the incidence of CWs in Indian water buffaloes, its association with BPV-2, and its successful transmission in the laboratory as well as natural hosts.

Keywords: BPV-2, buffalo papillomatosis, cutaneous warts, fibropapilloma, koilocytes

INTRODUCTION

Cutaneous papillomatosis (warts) in bovine is a contagious hyperplasia or benign neoplasm caused by BPV. 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 ten well characterized types: BPV-1 to -10, inducing papilloma and fibropapilloma (Shinichi et al., 2008). Recently, BPV-8 has been detected in European bison and BPV-7, 9 and 10 have been isolated from cattle in Japan (Ogawa et al., 2004; Ogawa et al., 2007; Hatama et al., 2008).

Cutaneous papillomatosis have been reported in cattle from around the world while in buffaloes there are only few sporadic reports and it is not a recognized disease entity. The aim of the 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

A survey was conducted in six organized dairy farms, local private dairies, large animal slaughter house, animal shows, etc. in U.P. and Uttarakhand, India, to record the incidence of CWs cases in buffaloes. The size, number, location of warts as well as age of animals and duration of affection were recorded. A total of 12 CW tissue biopsies and five blood samples were collected along...
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Table 1. Field survey for cases of bovine papillomatosis.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Organized Dairies</th>
<th>Breed and herd strength</th>
<th>No of Cases</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dairy Farm IVRI, Izatnagar</td>
<td>MB (460)</td>
<td>6</td>
<td>1.30</td>
</tr>
<tr>
<td>2</td>
<td>Private Local Dairies, Bareilly</td>
<td>MB (250)</td>
<td>8</td>
<td>3.20</td>
</tr>
<tr>
<td>3</td>
<td>Large Animal Slaughter House</td>
<td>MB (550)</td>
<td>4</td>
<td>0.73</td>
</tr>
<tr>
<td>4</td>
<td>Farmers Fair, IVRI, Izatnagar</td>
<td>MB (60)</td>
<td>2</td>
<td>3.33</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>1470</strong></td>
<td><strong>20</strong></td>
<td><strong>1.36</strong></td>
</tr>
</tbody>
</table>

Note: MB- Murrah breed.

with skin and blood samples from apparently normal buffaloes.

DNA analysis

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

Phylogenetic analysis

The PCR products were directly sequenced commercially on ABIPRISM dye terminator DNA Sequencing Facility at South Campus, Jawahar Lal Nehru University, New Delhi, India, 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 (DNASTAR Inc.).

Negative Staining and TEM

BPV was purified from CWs as described by Lancaster and Olson (1978). One mm² pieces of CWs were preserved in ice chilled 2.5% gluteraldehyde in 0.2 M phosphate buffer (pH 7.4) and stored at 4°C. Samples were processed and examined under an electron microscope. (Morgagni-268 and Philips CM-10, Holland) at the Electron Microscope Facility, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India.

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 a Fluorescence Activated Cell Sorter (FACS) calibur (Beeton Dickinson, San Jose, CA) at the Division of Biochemistry of the Institute. They were also examined for fluorescent pattern using the fluorescent dyes acridine orange-ethidium bromide (AO/EB, Sigma) and Hoechst (HO, Sigma) under a fluorescent microscope and the percentages of apoptotic cells were calculated.
Apoptotic index = (Total number of apoptotic cells / Total number of cells counted) X 100.

Experimental Transmission Studies
There were done in both natural as well as experimental hosts.

Hamsters
Seven adult male Syrian golden hamsters (*Mesocricetus auratus*), about 110-130 g in weight were used as infection and control groups containing four and three 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 six adult animals consisting of three cattle in Group I and three buffaloes in Group II were experimentally infected by multiple scarifications and subcutaneous inoculation on the neck, twice a week, with 1.0 ml of the 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 (Figure 1) and one case of teat papilloma were recorded in buffaloes. Grossly, warts were cauliflower-like with horny papillae or dome shaped with a smooth outer surface (0.5-2.0 cm in diameter) varying in number from one to six. Commonly affected sites were head, face, neck, legs and back. Most (14/20) of the cases were recorded in adult milking Murrah breed of buffaloes and only a few (6/20) cases were noticed in young animals. Bovine papillomatosis in cattle is a known disease while in buffaloes, only a few sporadic cases have been reported, and on the Indian subcontinent, it is an unrecognized entity (Joshi et al., 1994; Sood et al., 2006; Leishangthem et al., 2008a).

Etiological Studies

PCR
BPV-2 was detected in CWs (Figure 2) and lymphocytes of CWs affected animals. On phylogenetic analysis of the sequence of PCR products (Accession Nos: GQ369511.1 and GQ369510.1), 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 BPVs-like particles of approximately 50-55 nm in size against dark background (Figure 3). Earlier Leishangthem et al. (2008a) also observed scanty virus particles by
negative staining of CWs from cattle. TEM revealed 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. The cytoplasm had multiple, small vacuoles with pyknotic nucleus and small aggregates of electron dense PVs-like particles of about 50-55 nm in size (Figure 4-5). These finding are in accordance with those described for papillomatosis in cattle by earlier workers (Joshi et al., 1994; Dorte et al., 2004).

Pathological Studies
Gross and Histopathology

A total of 12 cases of CWs were studied in detail and two types of growth patterns were seen. Grossly, eight 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. The granular cell layer had prominent basophilic keratohyaline granules. Moderate to severe degrees of acanthosis (thickening of stratum spinosum) were observed with irregular rete pegs projecting deep into the dermis with lateral interconnections forming islands of dermal connective tissue surrounded by hyperplastic epidermal cells. The stratum spinosum revealed koilocytes, degenerating cells and intracytoplasmic eosinophilic inclusion bodies. The basal cell layer was hyperplastic with few mitoses. In the 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 outgrowths. Histopathologically, extensive hyperkeratosis and moderate to severe parakeratosis with basket weave appearance was seen in the stratum corneum. There was extensive acanthosis and uniformly downward growing columns of epidermal cells with thin cores of dermal tissue (Figure 6). The whole epidermal layer was hyperplastic with hyperchromatic nuclei and few mitoses in the basal cell layer. The stratum granulosa had intensively stained basophilic keratohyaline granules 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 for BP. However, fibropapilloma in cattle with acanthosis has been described by Lancaster and Olson (1982) with koilocytes, keratohyaline granules and inclusion bodies (Jelinek and Tachezy, 2005; Tomita et al., 2007) but it is for the first time an endophytic type of papilloma has been described and it further study is needed as such lesions were observed only in buffaloes and not in cattle.

Histochemistry (AgNORs count)

The AgNOR dots were in general large and round in CWs, and the average count was 1.9±0.43 whereas in malignant transitional cell adenocarcinoma, it was 3.9±0.34 indicating the relatively 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, an apoptotic index of 12-20 (AO/EB) and 8-15 (HO)
Figure 1. Cauliflower (arrow) like growth on fore leg.

Figure 2. Dairy, IVRI. Buffalo: L1-negative control, L2-L5 CWs, M-100bp marker.

Figure 3. Purified virus: virus-like particles (arrow) in buffalo Cutaneous Warts negative stain, EM x1,50,000.

Figure 4. Epidermocyte with pyknotic nucleus (pN) and crystalline array of virus particles in nucleus (N), EM x 4400.

Figure 5. Papilloma virus-like particles (arrow) of 50-55 nm (scale not shown) in size. EM x 3,00,000.

Figure 6. Acanthosis, uniformly downward growing columns of rete peges with no dermal fibrous hyperplasia. HE x100.
was recorded in CWs while that for normal skin was 3-5. With fluorescent staining, apoptotic live and dead cells could be differentiated 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, lentil-to pea-sized, nodular growths were noticed on the abdomen region in all the four animals in the infected group (Figure 7). The epithelium was intact with moderate to extensive proliferative changes in the dermal fibrous connective tissue. Fibroblasts and fibrocytes were seen with homogenous a glossy mass of eosinophilic collagen fibers in a radiating or whirling pattern (Figure 8). There was no encapsulation, and the 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 peanut-sized wart-like growths were seen along the line of scarifications on 60-70 DPI in both groups, in two cows and two buffaloes. In buffaloes, the lesions were similar to those in natural cases, both grossly and microscopically and were diagnosed as cauliflower-like exophytic fibropapilloma (Figure 9) and endophytic papilloma in buffaloes while fibropapilloma (exophytic, dome shaped) in cattle (Figure 10). This indicated that buffalo CWs are transmissible to both cattle as well as buffaloes. Experimental transmission of cattle CWs have been 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 hamsters as well as cattle and buffaloes and induce hyperproliferation of keratinocytes and fibroblasts. The lesions produced in hamsters were fibromatosis to fibroma where as in cattle and buffaloes fibropapilloma along with endophytic papilloma, which have not been described earlier. This is only the 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 percent of homology between the two. Also, cases of endophytic papilloma, observed in both spontaneous and experimental cases of CWs in buffalo need to be investigated further, as it is not common in cattle.

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REFERENCES


