INTRODUCTION
Papillomavirus is a common condition in cattle, usually benign in nature and characterized by small to medium sized growths on skin or mucous membranes. It is caused by bovine papillomaviruses (BPVs) which infect epithelial cells of skin or mucous membranes and produce hyperproliferative lesions. Thirteen different types of BPVs have been identified worldwide and these are said to be strictly species specific, but BPV1-2 can also infect equids, where it causes fibropapilloma, Real time PCR, Immunohistochemistry

Detection and Quantification of Bovine Papillomaviruses (BPVs) in Cutaneous Warts of Cattle and Buffaloes

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ABSTRACT
The present study describes the etiopathological characterization of cutaneous warts (CWs) of cattle and buffaloes. A total of 24 wart samples (cattle 15 and buffaloes 9) were studied. Grossly, most of CWs were of variable sizes, rough or coarse in texture, grayish/blackish/flesh colored, irregular in shape (dome or button) or resembling cauliflower-like masses and elevated from skin surface by broad base. Fibropapilloma was the most frequent histological type diagnosed. PCR was performed to detect the presence of BPVs and it revealed that CWs of I4 cattle were positive for BPV 2 while 3 samples were found to have mixed infection as they were also positive for BPV 1. Out of 9 CWs samples of buffaloes, 3 were found positive for BPV 1 and 3 for BPV 2. All the samples were negative for the BPV 5 & 10. Quantitative real time PCR revealed that DNA samples of cattle warts had comparatively higher viral load than those of buffaloes. Immunohistochemistry revealed that the PCNA and Ki67 immunopositivity was present in the basal and spinosum layer, respectively, of the fibropapilloma/papilloma. In conclusion, BPV types prevalent in the CWs of Indian cattle and buffaloes population are BPV 1 & 2 and Ki 67 may have association with viral replication as it expressed in spinosum layer where viral replication and assembly occurs.

Key Words: papillomavirus, cutaneous papillomatosis, fibropapilloma, Real time PCR, Immunohistochemistry


Kumar et al (2013). Bovine Papillomavirus in Cattle and Buffaloes

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MATERIAL AND METHODS

Cattle and buffalo CWs biopsies were collected from organized dairy farms i.e. Military Dairy Farm and Chak Gajaria, Dairy Farm, Lucknow; Referral Veterinary Polyclinic, IVRI, Izzatnagar, Buffalo Slaughter House, Bareilly and different villages of Dist. Bareilly, Raibareilly and Hardoi of Uttar Pradesh, Dist. Coonoor and Ooty of Tamilnadu and Dist. Sirmour of Himachal Pradesh. Biopsies were collected after giving local anaesthesia with lignocaine around the growth by veterinarians and preserved in 10% buffered formalin. A part of samples were also stored in sterile vials at 20°C for molecular studies.

Histopathology
After proper fixation in the 10% buffered formalin, tissues were cut into small sections with thickness of 2-3 mm and embedded in the paraffin by standard procedures. The paraffin embedded tissues were cut into 4-5 micron thick section and stained with hematoxylin and eosin as per conventional procedures (Culling, 1995).

Polymerase Chain Reaction
DNA was extracted from CWs samples stored at -20°C using the Genomic DNA Mini Kit (Qiagen). BPV was detected by PCR, targeting the L1 gene of BPV-1 & 2, 3'UTR region for BPV-5 and E2 gene for BPV-10 with specific primers. Oligonucleotide primers used in the study were commercially synthesized from Operon Biotechnologies, Genetix Biotec. Primers of BPV-1 (forward: 5'-ggg ggc gct cgt aat agg a 3'; reverse: 5'-atc ttc tgt tgg ggt gac g-3'), BPV-2 (forward: 5'-gtt atc cca gcc aac gaa gac cct gct aac tat agg a -3'; reverse: 5'-ctc ttt ctc ctt ctc-3'), BPV-5 (forward: 5'-gtc cgc ggg atc gga ct-3'), BPV-10 (forward: 5'-gtg gcc gcg gtc ggt atc gaa ct-3'; reverse: 5'-gga gcg cct gct aac tat agg a -3') were expected to amplify the specific viral DNA template of sizes 301, 165, 107 and 190 bp, respectively. Amplified DNA fragments were visualized by transillumination under UV light (Gelloc, USA) in 1.2% agarose containing ethidium bromide (0.5 g/ml) as per standard procedures. The PCR products were directly sequenced commercially on ABI-PRISM dye terminator at DNA Sequencing Facility, Division of Biochemistry, Delhi University, South Campus, New Delhi.

results

The CWs cases were observed on different parts of body of cattle and buffaloes (Table-1). Grossly, the CWs were having variable sizes, rough or coarse texture, grayish/blackish/flesh coloured, irregular (dome or button) or cauliflower-like masses and elevated from skin surface by broad and vascular base. One case of cutaneous papillomatosis in a cow bull showed abnormally extensive cauliflower-like growths on the neck and face with solitary warts on different parts of body (Fig. 1A). The warts in the heifers at Military Dairy Farm, Lucknow were restricted mainly on the ear pinna or around it and was characterized by multiple coalescing exuberant growths. After proper fixation in 10% buffered formalin, the tissue sections to microwave heat. After cooling the slides, sections procured from Qiagen as per standard procedures. The slides were washed with PBS and moist sections were covered with sufficient 3-Amino-9-ethyl-carbazole (AEC; Sigma Chemicals, USA) staining substrate for 10 minutes. The sections were then counterstained lightly for 3-5 min with Mayer's haematoxylin (Sigma, MHS 16). Slides were rinsed for 5 min in running tap water and mounted in glycerol gelatin. The fraction of immunopositive cells was counted as described previously (Woods et al., 1991) with some modifications.

Histopathologically, most of cases (cattle-9 and buffaloes-5) diagnosed as fibropapilloma (exophytic) consisted of moderate to extensive degree of cornification (hyperkeratosis) with basket wave appearance, varying degree of parakeratosis, hyperplastic stratum spinosum with presence of many koliocytes and islands of dermal connective tissue surrounded by hyperplastic epithelial cell layers. Basal cell layer was hyperplastic with hyperchromatic nuclei, mild mitotic activity and occasionally, invasive growth pattern was seen. Below epidermis, the neoepithelial stromal tissue consisted of large stellate shaped fibroblast cells and intense fibrocellular proliferative changes (Fig. 2A-C). Comparable to cattle (with abundance of koliocytes), buffalo warts showed only few koliocytes in the upper layer of stratum spinosum. Some cases showed the similar histopathological features except the fact that they had long rete pegs extending towards the fibrous stoma and proliferated there extensively (fibropapilloma endophytic). Other histopathological types diagnosed were papilloma occult/ fibroplastic type (Fig. 2D) and papilloma.
sequenced (HE603635, HE603636, HE600123 and HE600124) which revealed that the sequences generated in the present study has homology with earlier published sequences from India.

Table 1: Detection and quantification of BPV-1 & -2 in cutaneous warts of cattle and buffaloes

<table>
<thead>
<tr>
<th>S. No</th>
<th>Sample No</th>
<th>Species</th>
<th>Age (Years)</th>
<th>Sex</th>
<th>Location of growth</th>
<th>PCR results</th>
<th>Real Time PCR (Copy no./μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BPV-1</td>
<td>BPV-2</td>
</tr>
<tr>
<td>1</td>
<td>PCW-1</td>
<td>Cattle</td>
<td>8</td>
<td>M</td>
<td>Around scrotum and inner aspect of thigh</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>2</td>
<td>PCW-2</td>
<td>Cattle</td>
<td>7</td>
<td>F</td>
<td>Base of ear</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>3</td>
<td>PCW-3</td>
<td>Cattle</td>
<td>2</td>
<td>F</td>
<td>Ear pinna and at the base of ear</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>4</td>
<td>PCW-4</td>
<td>Cattle</td>
<td>2.3</td>
<td>F</td>
<td>Ear pinna</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>5</td>
<td>PCW-5</td>
<td>Cattle</td>
<td>2.5</td>
<td>F</td>
<td>Face and ear pinna</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>6</td>
<td>PCW-6</td>
<td>Cattle</td>
<td>3</td>
<td>F</td>
<td>Extensive growth on the ear pinna</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>7</td>
<td>PCW-7</td>
<td>Cattle</td>
<td>3.5</td>
<td>F</td>
<td>Ear pinna</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>8</td>
<td>PCW-8</td>
<td>Cattle</td>
<td>1.5</td>
<td>F</td>
<td>Cauliflower-like growth on whole body</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>9</td>
<td>PCW-9</td>
<td>Cattle</td>
<td>1.0</td>
<td>F</td>
<td>Nose and around eye</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>10</td>
<td>NCW-1</td>
<td>Cattle</td>
<td>4.5</td>
<td>F</td>
<td>Around the eye</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>11</td>
<td>NCW-2</td>
<td>Cattle</td>
<td>2.0</td>
<td>F</td>
<td>Left side of lower abdomen</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>12</td>
<td>NCW-3</td>
<td>Cattle</td>
<td>6.0</td>
<td>F</td>
<td>Lower eyelid</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>13</td>
<td>NCW-4</td>
<td>Cattle</td>
<td>5.5</td>
<td>F</td>
<td>Small cauliflower-like growth on the back</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>14</td>
<td>NCW-5</td>
<td>Cattle</td>
<td>3.0</td>
<td>F</td>
<td>Base of tail</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>15</td>
<td>NCW-6</td>
<td>Cattle</td>
<td>2.3</td>
<td>F</td>
<td>Neck</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>16</td>
<td>PK-1</td>
<td>Buffalo</td>
<td>7.0</td>
<td>F</td>
<td>At the base of ear</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>17</td>
<td>PK-5</td>
<td>Buffalo</td>
<td>1.5</td>
<td>F</td>
<td>Posterior aspect of thigh and at base of tail</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>18</td>
<td>PK-6</td>
<td>Buffalo</td>
<td>2</td>
<td>F</td>
<td>Two growths on the sacral region</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>19</td>
<td>PK-7</td>
<td>Buffalo</td>
<td>7</td>
<td>F</td>
<td>One growth at hump region</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>20</td>
<td>PK-8</td>
<td>Buffalo</td>
<td>7.5</td>
<td>F</td>
<td>Minute growths on back</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>21</td>
<td>PK-9</td>
<td>Buffalo</td>
<td>12</td>
<td>M</td>
<td>Growth on the third eyelid</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>22</td>
<td>PK-10</td>
<td>Buffalo</td>
<td>6</td>
<td>F</td>
<td>Wart-like growth on the hind leg</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>23</td>
<td>PK-11</td>
<td>Buffalo</td>
<td>2.5</td>
<td>F</td>
<td>Cauliflower-like growth above the base of tail</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>24</td>
<td>PK-12</td>
<td>Buffalo</td>
<td>2.5</td>
<td>F</td>
<td>Small slender growth on the lower back</td>
<td>+ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

Figure 1:
A) Cattle CW: Extensive irregular cauliflower-like growth on the face, neck and dewlap.
B) Cattle CW: Solitary, blackish, dome-shaped, growths on the ear pinna.
C) Buffalo CW: Cauliflower-like wart on the hind leg.
D) Buffalo CW: Cauliflower-like wart on the back.
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Kumar et al (2013). Bovine Papillomavirus in Cattle and Buffaloes

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Figure 2:
(A) Cattle CW: Papillary tip showing cornification and stratum spinosum with few koilocytes and connective tissue core. Fibropapilloma (exophytic), H&E × 100; (B) Cattle CW: Excessive cornification, epidermal thinning and elongated rete pegs penetrating in prominent dermal fibrous tissue. Occult papilloma/fibroblastic type, H&E × 100; (C) Buffalo CW: Excessive cornification, acanthosis of stratum spinosum, variable length rete pegs penetrating in hyperplastic fibrous connective tissue core. Fibropapilloma (exophytic), H&E × 40; (D) Buffalo CW: Mild cornification, hyperplastic fibrous connective tissue with presence of their islands within acanthotic stratum spinosum. Fibropapilloma (exophytic), H&E × 100.

Results of SYBR Green Quantitative PCR assay
CW DNA samples positive for BPV-1 & -2 were utilized for determination of DNA copy number present in them by using SYBR Green Quantitative PCR. These samples were tested together with known standard samples (dilutions of known concentrations of plasmid DNA) along with “No Template Control” (NTC). DNA samples of the CWs of cattle revealed a copy number of BPV-1 between 3.17E+03 to 4.85E+05 while in buffalo CWs range vary from 8.48E+02 to 3.41E+05. All DNA samples of the cattle CWs were positive for the BPV-1 and the copy numbers detected in them vary from 4.69E+03 to 1.91E+16. Five CWs samples of the ear collected from the Gazaria Dairy Farm and Military Dairy Farm, Lucknow showed very high copy numbers i.e. 2.66E+09, 5.78E+15, 6.22E+15, 1.91E+18 and 1.73E+18. Cattle CWs from Tamilnadu showed copy number of 5.35E+03 to 1.43E+04. Three DNA samples of the buffalo CWs showed copy number of 8.74E+04, 1.41E+04 and 1.41E+04.

Immunohistochemistry
CW samples were studied by IHC for PCNA and Ki67. A total of 9/17 (52.94%) CWs showed the immunopositivity for PCNA. Out of 10 fibropapilloma cases, only 4 (40%) showed immunoreactivity (PCNAmx 24.28±3.17; PCNAtot 15.02±8.83). Basal layer cells of the epidermis showed strong immunoreactivity while only few discrete cells in the parabasal and spinous layer showed weak immunostaining (Fig. 3C). Among 4 cases (cattle-3; buffalo-1) of fibropapilloma (endophytic), 2 cases of cattle showed the positivity for PCNA (PCNAmx 25.3±3.35; PCNA tot 14.45±6.42). One case each of cattle and buffalo of the occult papilloma/fibroblastic type were positive for PCNA and immunostaining was observed in the fibrous connective tissue (PCNAmx 22.33±2.64; PCNA tot 19.5±4.3). One papilloma case of buffalo was immunonegative for PCNA.

### Table 2: PCNA and Ki67 scoring in cutaneous warts

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Diagnosis</th>
<th>No.</th>
<th>PCNAmax</th>
<th>PCNAtot</th>
<th>Ki67max</th>
<th>Ki67tot</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Papilloma</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Fibropapilloma endophytic</td>
<td>4</td>
<td>20.5±3.35</td>
<td>14.45±6.42</td>
<td>22±0.0</td>
<td>12.5±0.0</td>
</tr>
<tr>
<td>3.</td>
<td>Fibropapilloma exophytic</td>
<td>10</td>
<td>24.28±3.17</td>
<td>15.02±8.85</td>
<td>18.5±1.5</td>
<td>13.43±4.34</td>
</tr>
<tr>
<td>4.</td>
<td>Occult papilloma / fibroblastic type</td>
<td>2</td>
<td>22.33±2.64</td>
<td>19.5±4.3</td>
<td>18±0.0</td>
<td>12.44±0.0</td>
</tr>
</tbody>
</table>

S. No.: Serial number; Diagnosis: Clinical diagnosis; No.: Number of samples; PCNAmax: Maximum PCNA staining; PCNAtot: Total PCNA staining; Ki67max: Maximum Ki67 staining; Ki67tot: Total Ki67 staining.
Ki67 immunopositivity was found in 4/17 (23.53%) samples, among them two were fibropapilloma (exophytic; Ki67max=22; Ki67tot=12.3), one each of fibropapilloma (endophytic; Ki67max=18.5±1.5; Ki67tot=13.45±4.54) and occult papilloma/fibroblastic type (Ki67max=18; Ki67tot=12.44). Immunostaining restricted mainly in the spinous layer while discrete immunopositive cells were also observed in basaland parabasal layer. Few cells in fibrous stroma also showed positive reaction.

DISCUSSION
Clinically, CWs were observed in organised farms as well as rural areas in the cattle and buffaloes. The CWs are known to be transmitted from cattle to buffaloes and vice versa (Singh and Somvanshi, 2010). In Military Dairy Farm, Lucknow, UP warts was confined mostly to ear pinna which may be due the fact that these were transmitted from the infected animal to healthy one by tattooing machine. Association of tattooing with papilloma development on the ear of calves were reported earlier (Studdert et al., 1988). Histopathology revealed that fibropapilloma (exophytic) was the most common histopathological type observed in CWs of both cattle and buffaloes. Fibropapilloma (endophytic), papilloma occult/ fibroblastic type and papilloma were other types found in CWs. Similar histopathological types were also reported by earlier workers in the CWs (Pangty et al., 2010). Grossly, it was observed that few CW cases of cattle spread extensively to the adjacent areas forming large cauliflower-like masses but in buffaloes, almost all cases showed solitary growths. This finding was observed earlier also (Somvanshi, unpublished data).

PCR results revealed that DNA of CW of cattle was positive in 14 cases for BPV-2 while 5 were found to be positive for BPV-1. Five CW cases showed mixed infection as they were positive for both BPV-1 & -2. Out of 9 CW cases of buffaloes, 5 were found positive for BPV-1 and 3 for BPV-2. All samples were negative for BPV-5 and -10 which indicated that hairy skin is less prone to infection by these viruses although in earlier studies BPV-5 has been reported from the teat and facial skin (Lindholm et al., 1984; Bloch et al., 1994) and BPV-10 from the teat warts (Hatama et al., 2008; Rai et al., 2011). BPV-1, -2 and their mixed infections were also detected by previous workers in cattle (Leishangthem et al., 2008; Pangty et al., 2010; Pathania et al., 2011) and buffaloes (Silvestre et al., 2009; Singh and Somvanshi, 2010; Pangty et al., 2010) indicating that these two BPV types are prevalent in Indian cattle and buffalo population.

Real time PCR revealed a wide range of virus load in different samples for both BPV-1 & -2. Cattle CWs showed comparatively high viral DNA load of BPV-2 than buffaloes.
while for BPV-1 the CWs of both cattle and buffaloes had almost similar range. Earlier researchers also quantified the viral load of BPV-1 & -2 in cattle and buffalo CWs (Pangty et al., 2010; Nagarajan, 2011) but a comparative study is lacking. Determination of viral load and expression of the BPV E2, E5, E6 and E7 genes in four clinical types of equine sarcoid using quantitative real PCR was performed earlier (Bogaert et al., 2007) and found that nodular sarcoid showed a significantly higher viral load than the other types.

In present study, PCNA positivity was observed mainly in basal cells and few discrete cells of parabasal and spinous layers of fibropapilloma and occult papilloma/leukoplakia. Similarly, PCNA was predominantly detected in the basal layer of the epidermis and in the superficial dermis in fibropapilloma of linea alba and teats in four heifers and the highest number of PCNA-positive nuclei was found in the basal layer of the epidermis (Jelínek and Tachezy, 2005). Lesions produced by human papilloma virus showed extensive PCNA expression in the epidermis, including the basal, parabasal and spinous layers (Penney et al., 1992; Lu et al., 1993; Ozsoy et al., 2011). Ki67 immunopositivity was restricted mainly in the spinousum layers, although few positive cells were also detected in the basal and parabasal layer of the epidermis. Few comparative studies on the expression of PCNA and Ki67 in relation to HPV indicated that the expression of PCNA restricted mainly to the basal and parabasal layers while Ki67 was detected mainly in the spinous and upper layers of the epidermis along with HPV (Boon et al., 1993; Lu et al.,1996; Lu et al., 1999). Such findings were interpreted as Ki 67 expression was needed for the viral (HPV) replication and induction of both the markers were independent (Boon et al., 1993). Although present investigation also revealed similar expression behaviour of the PCNA and Ki67 in the bovine papillomatisis cases but to interpret the result in relation with BPV further studies needed.

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