3.1. Biological specimens

Wart biopsy materials from clinically positive wart cases in both bovine and bubaline species that suffered from spontaneous cutaneous papillomatosis throughout West Bengal, irrespective of organized and unorganized sector, during the study period from August 2008 – January 2013, served the source of specimens for the present study. Cases bearing clinically ambiguous (papillomatous or wart like) lesions were also biopsied for examination and diagnosis for BPV. To minimize the risk of cross contamination between different lesions, the following precautions were taken: 1) each sample was collected by hand (wearing gloves, changed for each sample) and packed individually and maintained at 4 °C until the DNA extraction procedure was completed; and 2) all materials used in the analysis were sterilized and disposable. Fragments from each cutaneous wart (CWT) were triturated in phosphate buffered saline solution (pH 7.2), and the suspensions (10-20% w/v) were centrifuged for 15 min at 3000 x g at 4°C. Aliquots (250µl) of the supernatant were treated with lysis buffer (1o MTris; 1 Mm EDTA; 0.5% Nonidet P 40; 1% SDS and 0.2 mg/ml proteinase K) (Invitrogen, Life Technologies, USA). After homogenization, the samples were incubated at 56°C for 30 min. Fractions from each sample were taken for DNA extraction. Samples collected from Slaughter houses were also preserved in 10% buffered formalin.

3.1.1 Field Survey for clinical and epidemiological examination

To study etiopathology of cutaneous papillomatosis in cattle and buffaloes, cutaneous samples (skin and teat) lesions/ warts/growths were intended to investigate in relation to the presence of different types of BPVs and gross and microscopic lesions. The present investigation was designed to study the patho-morphology and to detect the BPV types involved in development of wart like lesions on skin and teats of cattle and buffaloes. Field survey was conducted both in organized and unorganized dairy farms, private dairies, goshalas, slaughter houses, cattle markets, Veterinary clinics and even at farmers door-step both at rural and urban livestock owners including animals grazing fields(pasture lands) covering vast areas of tropical West Bengal. All cases were subjected to clinical examination and the shape, size, number, types and location of the papillomas were recorded. The records of cows in relation to age, sex, breed; location and duration of warts/ papillomas were also noted. History, clinical details (temperature, pulse, respiration, heart rate etc.), ectoparasitism, gross appearance of the warts, duration of infection, and treatment attempted if any, were also taken into consideration. The suspected infected cattle were visually examined for detection of external parasites and coprologically inspected for internal parasites and their eggs. Ticks found on the cattle skin were identified macroscopically. Internal parasites were diagnosed by
faecal examination. Faecal matters were collected by back racking from BP infected cattle and were examined by concentration flotation technique.

### 3.1.2 Tissue samples

Cattle Cutaneous wart biopsies were collected from different organized dairy farms of West Bengal: State Livestock Farm, Kalyani; Haringhata Dairy Farm, Cattle section of Haringhata Farm, Haringhata, Nadia; Bull Mother Farm, Haringhata, under Paschim Banga Go Sampad Bikash Sangstha; NDRI Eastern Regional Station Dairy, Kalyani, Nadia; different societies and Goshalas under Calcutta Pinjrapole society(CPS), CPS-GoshalaLilua, Howrah; CPS-Sodepur Goshala, North 24 Parganas; CPS-Anandnagar, Kalyani branch, Nadia, CADC, Sonamukhi, Bankura, Ashram Dairies of Ramkrishna Mission, Narendrapur, Kolkata, Nigamananda Ashram dairy, Halishar, North 24 Parganas, Shantashram, Dattapukur, N-24 Pargana, Large animal slaughter houses both from Tangra and Metiaburuj, Kolkata Municipality, Slaughter house in DGHC, Darjeeling; certain Veterinary Dispensaries including BAHC, ABAHC and SAHC in Nadia; Private dairies, both small scale and large scale, those convinced to take tumour biopsy, from field level sporadic cases of warts and cattle markets at Puranpani (Ghora Dhara Hat), Ranibandh, Bankura, Bankura. Wart tissue samples were also collected from the sheds of livestock owners from all other districts during entire survey work, of course from the willing ends for giving consent to extirpate warts for taking biopsy material.

Buffalo cutaneous wart biopsies were collected from NDRI experimental animal shed, Kalyani, Eastern Regional Station, Municipality Slaughter Houses of Darjeeling and Kolkata (Pilkhana/Tangra and Metiaburuj), Rural livestock farmers of Halishar, Kanchrapara, Kankinara, Naihati of North 24 Pargana, Patrasayer, Nabason, Parulia, Bijipur of Patrasayer Block, Bankura, Naldanga and Khandaghosh of Burdwan, Jhulonia, Saheb-bagan of Hooghly, Bhabanipur of Kalyani, Kampa, Haringhata of Nadia, organised Government Dairy Farm, SLF, Kalyani, Haringhata Farm.

Strict aseptic precautions were followed during sample collection. Area thoroughly cleaned with Lugol’s iodine or antiseptic alcoholic solutions and cutaneous warts were collected under local anaesthesia and immediately stored at 4°C. The blood, dusts or any other contaminants were properly removed from the sample. A part of each sample showing gross lesions was stored in sterile vials at -20°C for molecular studies.

### 3.1.3. Wart lysate preparation

Wart biopsies as collected, were sectioned (1-2 mm3), ground in phosphate buffered saline solution (PBS pH 7.2), transferred into 100 µL phosphate buffered saline (PBS) and treated for 15 minutes at 95°C. The suspension (20% w/v) was centrifuged for 15 minutes (600 x g) and the supernatant was stored at -20°C.
3.1.4 Glass and Plastic Wares

The glass wares used in the present study were procured from Borocil India. Glass wares were cleaned as per standard procedures and autoclaved. All the plastic wares used in the present study were procured from M/S Axygen Scientific Private Ltd. (India).

3.1.5 Equipment and Instruments

All the standard equipment and instruments available in Divisional laboratories, Department of Microbiology, Zoology and Biochemistry, Kalyani University, Electron Microscope Facility, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, and National Institute of Cholera and Enteric Diseases, Kolkata, and Bose Institute, Kolkata. Specifications of the instruments/equipment are given at appropriate places in the text, wherever necessary.

3.1.6 Buffers, Media and Reagents

The chemical composition of all the buffers and reagents used in the present study are listed in the Appendix. These buffers and reagents were prepared in DNAse and RNAse free water.

3.1.7 Chemicals, Molecular Biology Reagents and Enzymes

All the chemicals, molecular biology reagents and enzymes used in the present study were obtained from reputed firms such as Ms Sigma, MBI Fermentas, QIAGEN, Operon Biotechnologies, Genei, Promega and Life Technologies. Wherever necessary, Molecular Biology grade chemicals and biochemical were used. Specification of the chemicals, molecular biology reagents and biochemical are given at appropriate places in the text, wherever necessary.

3.1.8 Molecular Biology Kits

For extraction of DNA, QIAamp DNA Mini Kit (QIAGENS, USA) PCR Master Mix, SYBER Green PCR Master Mix (QIAGENS, USA), Mini Elute PCR Purification kit for purification of PCR products, was used. Cloning and Plasmid DNA isolation was done by using Zyppy TM Plasmid Mini Prepararation kit (Zymo Research).

3.1.9 Oligonucleotide Primers

Oligonucleotide primers used in the present study were got commercially synthesized from Integrated DNA Technologies, and Operon Biotechnologies, Genetix, Biotec Asia Private Limited. The nucleotide sequences are given below:

BPV-1
1. 5´-gga gecctgtaac tat agg a-3´ (Forward)
2. 5´-atc tgttgtgggtggt gac-3´ (Reverse)
**Materials and Methods**

**BPV-2**
1. 5´-gtt ataccaccaagaagaccct -3´ (Forward).
2. 5´- ctggtttgcaacagctctcttc -3´ (Reverse)

**BPV-5**
1. 5´-atg gcggtttgg cag cagcagcaa gg-3´ (Forward).
2. 5´-ccc agcagaccccct tat tt-3´ (Reverse)

**BPV-9**
1. 5´-tgt cat taa tat tattcagca ag-3´ (Forward).
2. 5´-ttc attataacc act gtcgtc -3´ (Reverse)

**BPV-10**
1. 5´ctc tag ggagaaagtctcg -3´ (Forward)
2. 5´-tct gac act ctcgaaagagg -3´ (Reverse)

These primers amplify a L1 gene DNA fragment of 301bp for BPV-1, 165bp for BPV-2, 961bp for BPV-5.

**Properties and oligonucleotide specification**

**BPV-1, F**, DNA Bases-22 Tm(50m NaCl):58.9°C, GC Content-54.5%, Mol. Wt. 6744.4.

**BPV-1, R**, DNA Bases-21. Tm (50m MNaCl):55.5°C, GC Content-47.6%, Mol. Wt. 6514.3.

**BPV-2, F**, DNA Bases-24. Tm( 50m NaCl):52.1°C, GC Content 42.8%, Mol. Wt. 63432.

**BPV-2, R**, DNA Bases-24. Tm (50m MNaCl):57.9°C, GC Content 52.1%, Mol. Wt. 6941.5.

**BPV-5, F**, DNA Bases-26. Tm (50m MNaCl):68.0°C, GC Content 61.5%, Mol. Wt. 8101.3.

**BPV-5, R**, DNA Bases-20. Tm (50m MNaCl):57.1°C, GC Content 55.5, Mol. Wt. 5972.9.

**BPV-9, F**, DNA Bases-20. Tm (50m M NaCl) : 45.4°C, GC Content 30%, Mol. Wt. 6115.0.

**BPV-9, R**, DNA Bases-21. Tm (50mM NaCl): 49.9°C, GC Content 38.0%, Mol. Wt. 6331.2.

**BPV-10, F**, DNA Bases-20. Tm (50 mMNaCl):51.6°C, GC Content 50%, Mol. Wt. 6157.0.

**BPV-10, R**, DNA Bases -20. Tm (50mM NaCl) :53.5°C, GC Content 50%, Mol. Wt. 6126.0.

**3.1.10 PCR amplification of target gene**

Both conventional and Real time PCR (with DNA- binding dye (SYBR Green 1) were performed for BPV typing and analysis. In conventional PCR, the amplified product is confirmed by gel electrophoresis and image analysis whereas by the real time PCR sample could be detected very fast and specific. In the Real-time PCR assay, a positive reaction was detected by accumulation of a fluorescent signal.
In Real-time, the DNA–binding dye is SYBR Green 1 which binds non-specifically to double stranded DNA and it increases up to 1000-fold. From the reaction, the overall fluorescent signal is directly proportional to the amount of double stranded DNA. Therefore, the cycle threshold (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold.

3.1.11 Soft wares used for phylogenetic analysis

The soft wares used for sequence comparison and phylogenetic analysis were EDITSEQ and MEG ALIGN modules of Laser gene software (DNASTAR Inc.).

3.1.12 Bovine Papilloma virus (BPV-1 and BPV-2) partial and complete genomic sequences

Partial and complete genomic sequences of L1 region of BPV-1 and BPV-2 as available till date were downloaded from EMBL and NCBI, an internet public database utility.

3.2. Methodology

3.2.1 Studies on spontaneous cases of warts

Field survey: Random field survey was conducted during the period from August 2008 to January 2013 at rural livestock holders in villages of all nineteen districts of WB covering organised dairy farms, Veterinary institute dairy farms, Veterinary clinics, slaughter house, goshalas, cattle markets, etc. to record the incidence of the disease among the cattle and buffalo population. The size, number, type, duration of affection and location of warts were recorded. Field and farm level survey were made as per following format.

Name and address of the dairy farm, breeds of animals maintained in the farm (exotic, Indian, crossbred and upgraded), total number of animals in the herd (number of male and number of female calves, number of heifers, number of cows, number of bulls), herd having cases of cutaneous warts and teat warts: yes/ no, if yes then number of calves affected, number of heifers/ bull calves affected or number of cows or bulls affected, number of CWTs occurring: mild (1-10), moderate (>10 and up to 50), extensive (>50 and may be up to 100 and more), warts located on which anatomical region, type of warts occurring in dairy animals: broad base with rough surface, pedunculated with rough surface, rice grain like and numerous, extensive, complicated and bled due to trauma, losses occurring due to warts: loss of body weight, loss of milk production, affection of udder and teat causing hindrance in milking and mastitis, causing interference in breeding/coitus, trauma of wart resulting in infection and dermatitis, treatment attempted: surgical intervention, Alopatic medication like use of Injectable Antimony compound, oral and injectable Ivermectin, oral and Injectable Levamisole, Homeopathic medicines like the administration of sulphur and Thuja,
Autohemotherapy, auto-immunization or auto-genous vaccination and no treatment or regressed automatically, any other information like material collected in formalin, material collected in glycerine, treatment given (specify) and follow up progress, date and place.

3.2.2 Clinical studies

The animals both cattle and buffaloes covering a population of thirty four thousand six hundred fifty six were examined clinically for the presence of wart like lesions on the animal’s body in field condition. Besides screening of animals at owner’s doorstep, animals at Government dairies, Goshalas, private dairies, slaughter house cattle markets and private organized dairies were also examined for warts incidence. Recorded data were collected from Government Veterinary clinics of the state, under the Directorate of Animal Husbandry and Veterinary Services, West Bengal.

3.2.3 Haematological and serum biochemical studies

Approximately, 3 ml of blood samples from individual animals (were collected in dry sterilized vial containing anticoagulant, EDTA @1mg/ml. The haematological parameters, such as total erythrocyte count (TEC), haemoglobin concentration (Hb), packed cell volume (PCV), total leucocyte count (TLC) and differential leucocyte count (DLC) were carried out as per Jain, 1986. For this purpose blood was collected from 22 numbers of clinically affected cattle and 32 numbers of ailing buffaloes randomly.

For serum biochemical study, about 5ml of blood from both cattle and buffalo were collected in test tubes and allowed to clot in a slanting position. The clot was broken from sides after 2-3 hours, centrifuged at 1000rpm for 10 minutes and supernatant was collected by Pasteur’s pipette and stored at -20°C until further use. Plasma was obtained after centrifugation of unclotted blood and was carefully removed and stored at -20°C. Biochemically, the sera samples were analysed for total protein (Biuret method), albumin (BCG Dye binding method), cholesterol (Wybenga and Pileggi One step method) and BUN (DAMM method) using standard commercial diagnostic kits.

3.2.4 Pathological studies

Lesion in situ was thoroughly cleaned with Lugol’s iodine and biopsied surgically under local anaesthesia and all contaminants from CWT tissues were properly removed. Collected samples were preserved immediately with proper fixatives for both histopathological as well as ultra-structural studies.

3.2.5 Histopathology

The neoplastic lesions were totally or partially removed by surgical incision or punch. The samples in copies were conserved appropriately for tissue processing by histopathology.
For histo-pathological studies the tissues were preserved in 10% buffered formalin. Formalin fixed tissues were processed routinely and embedded in paraffin. Paraffin embedded tissues were cut into 4-5μm thickness and stained with haematoxylin and eosin as per procedure of (Culling, 1975). Stained slides were examined under microscope and observations recorded.

3.2.6 Ultra-structural Studies

Immediately after surgical excision few portion of the sample was fixed in 2.5% gluteraldehyde. Wart tissue samples of both cattle and buffaloes were preserved as recommended and processed for both transmission (TEM) as well as scanning electron microscopic (SEM) studies, respectively at the Electron Microscope (Morgangni 268 and Philips CM-10, Holland), Facility Centre Department of Anatomy, All India Institute of Medical Sciences, New Delhi and in the Electron Microscope Unit of National Institute of Cholera and Enteric Diseases(NICED), Kolkata and SEM in sophisticated analytical instrument facility (SAIF) in Bose Institute, Kolkata respectively. The samples were screened for suitable areas and ultra-structural images were saved using Soft Imaging Viewer software.

3.2.7 Scanning Electron Microscopy

Scanning electron microscopy was done according to the following standard procedure. Small pieces of infected tissue (cutaneous wart and teat wart) as excised were cut into further small pieces approximately 0.5 to 1 cm size. Tissues were rinsed properly with NSS and fixed in Karnovsky buffer (as fixative). After fixation the tissues were subjected to dehydration with graded ethanol and amyl acetate. Tissues were then passed through the critical point drying in liquid carbondioxide. After critical point drying the tissues were coated with gold/palladium (called sputter coating). The tissues were then ready for SEM studies. The healthy cutaneous tissues were also processed in the same way and were examined under the SEM.

3.2.8 Transmission Electron Microscopy

For transmission electron microscopic (TEM) studies, freshly collected warts, normal skin and teat skin samples were preserved as pieces of 1 square mm size in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH7.4) at 4 °C. The tissue pieces were then washed with three changes (2 hours each) of cold 0.2 M phosphate buffer (pH7.4), fixed with 1% Osmium tetroxide for four hours at 4°C, dehydrated in ethyl alcohol, cleaned and embedded in epon-araldite. Sections were cut with glass knives on a Porter-Blum ultramicrotome (Ultracut Reichert-Jung, Austria) and mounted on copper grids and stained with uranyl-acetate (Watson, 1958), subsequently by lead citrate (Reynolds,1963). They were washed and allowed to dry on a filter paper in a covered Petridis at room temperature. The grids were viewed in EM (Morgani-268) at Electron Microscope Facility, National Institute of Cholera and Enteric Disease (NICED), Kolkata, West Bengal.
3.2.9 Negative Staining

This was performed with partial modification of the technique according to the methodology described by Brenner and Horne (1959), Hayat and MILLER (1990) and Madely (1997). Skin lesion fragments were suspended in phosphate buffer 0.1 M and pH 7.0. Wart tissue lysate as prepared were preserved in chilled 2.5% gluteraldehyde in 0.2 m phosphate buffer (pH7.4) and stored at 4°C. The fixed sample was then applied to a coated grid for TEM. A drop of the suspension was put on a sheet of parafilm and a formvar-carbon coated 400m metallic copper grid was kept over it (filmed side down) for at least 1 minute. Grid was then removed and excess liquid was allowed to drain off by touching edge to a piece of clean filter paper. The grid was placed over a drop of 2% Phospho-tungstic acid (PTA) stock as above, drained off excess fluid as before and allowed to dry for a few minutes and then examined under an electron microscope (Morgagni-268 and Philips CM-10, Holland) in the above mentioned TEM Facility, NICED, Kolkata, West Bengal.

3.3 Polymerase Chain Reaction (PCR) analysis of wart samples

3.3.1 DNA Extraction from Papillomatous Lesions (extraction of total DNA)

Total DNA was extracted from all tissue samples using the DNeasy tissue kit (QiagenInc, Valencia, California, USA) according to the protocol recommended by the manufacturer. Working in a laminar flow cabinet equipped with an HEPA filter, approximately 25 mg of each papillomatous skin (wart) lesion was minced with a sterile surgical blade and placed and ground in a sterile 1.5 ml micro-centrifuge tube. The tissues were incubated overnight at 55 °C in a mixture containing 180µl of digestion buffer ATL and 20µl proteinase K (20mg/ml) until lysis was complete. Then, 200µl of buffer AL and 200µl of 100% molecular grade ethanol were added to precipitate the DNA. The solution was then centrifuged in a DNeasy Spin Column to bind the DNA to the membrane and the membrane was washed with 500µl of buffers AW1 and AW2 for 1 minute each time. A final centrifugation step was performed to eliminate residual ethanol remaining in the membrane. The DNA was eluted in 200 µl of buffer AE and evaluated for yield and purity by spectrophotometry using the ultrospec 3000. A negative tissue sample from uninfected cattle and buffalo skin tissue samples was extracted along with each set of tissue samples for use as a negative control in PCR analyses. The eluted DNA samples were stored in 1.5-ml screw-cap tubes at-80°C.

3.3.2 PCR Reaction

Total DNA extracted from cutaneous warts suggestive of papillomavirus infection was assayed by PCR targeting the L1 major capsid protein gene with specific primers of BPV-1,-2,-5, -9&-10 types as stated earlier.
For PCR reaction mixture was prepared in a final volume of 50µl containing the following mixture:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PCR Mix</td>
<td>25µl</td>
</tr>
<tr>
<td>2. Primer(Forward + Reverse)</td>
<td>3µl</td>
</tr>
<tr>
<td>3. DNA Template</td>
<td>5µl</td>
</tr>
<tr>
<td>4. Nuclease free water to make final volume of 50µl</td>
<td>17µl</td>
</tr>
</tbody>
</table>

The contents were mixed by moderate vortexing and brought down to the bottom of the tube by brief centrifugation. The tubes were put into the thermal cycler (Eppendorf, USA) and following thermal profile and cycling condition were used for PCR reaction.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Conditions</th>
<th>BPV-1 and BPV-2</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial denaturation</td>
<td>94°C for 3 min</td>
<td>35</td>
</tr>
<tr>
<td>2.</td>
<td>Denaturation</td>
<td>94°C for 40 sec</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Annealing</td>
<td>50°C for 40 sec</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Elongation</td>
<td>72°C for 1 min</td>
<td></td>
</tr>
</tbody>
</table>

A final cycle incorporated an extension at 72°C for 10 min and hold at 4 °C till retrieval from the thermal cycler.

3.3.3 Confirmation of the PCR amplicons

Small aliquots of the amplified DNA fragments were resolved by horizontal electrophoresis in 1.5% agarose containing ethidium bromide (0.5g/ml) and visualized by trans-lumination under UV light using a gel documentation system (GELDOC, USA) as per standard procedures and the results were recorded. For size comparison, a 100 bp molecular weight DNA marker (MBI, Fermentas) was electrophoresed in parallel to the PCR amplicons.

3.3.4 Cloning of PCR amplicons

3.3.4.1 Purification of PCR amplicons

The purification of the PCR amplicons was performed using Min Elute PCR Purification Kit; QIAGENs as per manufacture’s recommended protocol described below:

1. 5 volumes of buffer PB was added to 1 volume of the PCR reaction and mix.
2. A Minelute column was placed in a provided 2ml collection tube in suitable tubes.
3. To bind DNA, the sample was applied to the Minelute column back into the same tube.
4. Flow-through was discarded. The Minelute column was placed back into same tubes.
5. To wash 750 ml Buffer PE was added to the Minelute column and centrifuged for 1 minute.
6. Flow-through was discarded and minelute column was placed back into the same tube. The column was centrifuged for an additional 1 min. at maximum speed.
7. The Minelute column was placed in a clean 1.5 ml micro-centrifuge tube.
8. To elute DNA, 10 ml Buffer EB (1.0Mm Tris. Cl, Ph 8.5) or water was added to the centre of the membrane and let the column stand for 1 min and then centrifuged for 1 min.

3.3.4.2 pGEMT easy cloning reaction (Ligation of purified PCR amplicons)

The purified amplicons were ligated to the Pgemt-easy vector. The following reagents were added in a 500µl microfuge tube placed on ice, to set up a 10µl ligation reaction. The contents are Purified PCR product- 3µl, 2 x T4 DNA ligase Buffer- 5µl, T4 DNA ligase enzyme- 1µl, pGEMT-easy vector- 0.5µl, Nuclease free water to make- 10µl, and the contents were mixed by gently tapping the tube followed by incubation at 16°C in a water bath for overnight incubation.

3.3.4.3 Preparation of competent cells

Competent E. coli strain DH5α cells were used for the transformation. The cells were made competent as follows:
1. Frozen culture of E. coli strain DH5α was revived by growing at 37°C overnight in 3ml LB broth.
2. Next day, 200µl of freshly grown E. coli DH5α cells were inoculated in 20 ml LB medium. The cells were again incubated at 37°C under constant shaking until OD600 was between 0.25 and 0.35. At this stage, the growth of the cells was stopped by placing them on ice.
3. The cells were thereafter pelleted by centrifugation in a refrigerated centrifuge at 6000 rpm for 10 minute.
4. The cell pellet obtained was re-suspended in 1/10 volume (2ml) of TSS and kept on ice for 1 hour. The cells were then aliquot in 1.5 ml microfuge tubes (200 ml each) and preserved at -70°C until used.

3.3.4.4 Transformation of competent cells

1. The ligated product in a 4 µl volume is diluted with TCM to make final volume to 200µl and kept on ice for 30 min.
2. It was then mixed with 200ml of the competent cells and incubated on ice for 2 hrs.
3. Thereafter, the cells were subjected to heat shock at 45°C for two min. followed by immediate chilling on ice for 5 min.

4. SOB medium (600 ml) was added to each tube and the cells were incubated at 37°C for 1 hr with constant shaking.

5. The transformed cells were then plated on LB agar plates containing ampicillin (20 mg/ml) and incubated overnight at 37°C.

6. The L-B agar plate was checked for the presence of colonies.

7. The plates with recombinant colonies were stored in 4°C after proper labelling till further use.

3.3.4.5 Screening of recombinants

Single bacterial colony was taken in tubes containing 3 ml of LB broth with ampicillin (20mg/ml), and tubes were incubated overnight at 37°C in incubator shaker at 200 rpm.

3.3.4.6 Plasmid DNA isolation

The standard alkaline lysis Miniprep method was employed for plasmid isolation. Briefly, the method is presented below:

1. 1.5 ml of the culture was transferred to a sterile microfuge tube and the cells were pelleted by centrifugation at 12000 rpm for 2 min and supernatant was discarded.

2. 300 ml of P1 was added to re-suspend the cells.

3. 300 ml of P2 was added and mixed gently.

4. Immediately, 300 ml of chilled P3 was added and mixed gently and incubated in ice for 5 min.

5. Centrifugation was done at 10000 rpm for 10 min at 4°C

6. The supernatant was collected in fresh micro-centrifuge tubes.

7. Equal volume of phenol: chloroform: iso-amylalcohol (25:24:1) was added and mixed well.

8. Centrifuge at 10000 rpm for 10 min at room temperature.

9. The upper aqueous layer was taken in a fresh test tube.

10. The 0.6 volume of isopropanol was added and mixed gently and centrifuged at 10000 rpm for 15 min at room temperature.

11. The supernatant was discarded.
12. The pellet was washed with 1 ml 70% chilled ethanol and centrifuged at 10000 rpm for 1 min at 4°C.
13. The pellet was air dried.
14. The pellet was re-suspended in 30 µl Tris-EDTA (10:1) buffer.
15. It was then incubated at 37°C for ½ an hour.
16. The DNA sample was kept at 4°C.

3.3.4.7 Digestion of plasmid DNA

Plasmid DNA was digested using EcoR1 enzyme using the following reaction for 10 µl:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>3 µl</td>
</tr>
<tr>
<td>10 x EcoR1 buffer</td>
<td>1 µl</td>
</tr>
<tr>
<td>EcoR1 enzyme</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.5 µl</td>
</tr>
</tbody>
</table>

Following completion of restriction digestion, total restriction digest was run on 1.5% agarose gel containing ethidium bromide along with 100 bp DNA marker. The positive recombinant clones were identified and stored at 4°C after proper labelling, until further use.

3.3.4.8 Sequencing of PCR amplicons

The cloned PCR amplicons were got commercially sequenced on ABI-PRISM dye terminator DNA sequencing facility using T7 sequencing and the sequenced data generated was received as coloured electro-pherograms and text files.

3.3.4.9 Analysis of sequence data and phylogenetic analysis

Using EDITSEQ and MEGALIGN modules of LASERGENE software (DNASTAR Inc.), the published and sequenced data were aligned by the clustal method. Wherever necessary, the nucleotide sequences were truncated to 301bp and 165bp lengths for L1 region of BPV-1 and BPV-2 respectively. The sequenced data was compared with published sequences (EMBL and NCBI) of BPVs.

The standard nucleotide alignment output and percent similarity and divergence values based on nucleotide sequences similarities were obtained using MEGALIGN module.

Aligned nucleotide sequences 301bp for L1 region of BPV-1 and 165 bp for L1 region of BPV-2 were subjected to phylogenetic analysis using MEGALIGN module as per standard procedures.
3.4 Quantitative SYBER Green PCR Assay

Quantitative SYBR Green PCR assay was performed on cutaneous warts using Strata gene MX-3000P real time PCR cycler using commercial reagents procured from Qiagen as per manufacturer’s recommendations. PCR reaction comprised of 25µl of 2.5 x SYBR Green PCR Master mix, 0.6 µl (60 p mole) each of sense and anti-sense primer, 3.0 µl DNA and nuclease free water to make a total reaction volume of 50µl. The reaction mixture was mixed by vortexing, centrifugation and transferred to optical- grade strip- capped 200µl PCR tubes. Reactions were set in for each sample for SYBR Green PCR and light exposure was minimised or avoided.

The thermal profile for BPV-1 comprised of three segments. Segment -1 comprised of initial denaturation at 95°C for 15 minutes followed by Segment-2 consisting of 40 repetitive cycles of denaturation at 94°C for 15 seconds, annealing at 51°C for 30 seconds and extension at 72°C for 30 seconds each. Dissociation curve analysis was performed in segment -3 which comprised of a single cycle of denaturation at 95°C for 30 seconds. For generation of amplification plots, fluorescence value (dR) was collected at second plateau (annealing phase) and to confirm fidelity of amplification, dissociation curve analysis was performed at the end of the run.

The thermal profile for BPV-2 comprised of three segments. Segment -1 comprised of initial denaturation at 95°C for 15 minutes followed by Segment -2 consisting of 40 repetitive cycles of denaturation at 94°C for 15 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 30 seconds each. Dissociation curve analysis was performed in segment-3 which comprised of a single cycle of denaturation at 95°C for 1 minute, partial renaturation to 58°C for 30 seconds and denaturation again at 95°C for 30 seconds. For generation of amplification plots, fluorescence value (dR) was collected at second plateau (annealing phase) and to confirm fidelity of amplification, dissociation curve analysis was performed at the end of the run.

3.4.1 Purification of PCR products

For preparation of the standard curve BPV-1 and BPV-2 purified DNA was obtained by purifying PCR product by QIAquick Gel extraction kit methods.

For the purification of PCR products, the following protocol was followed briefly,

1. The DNA fragment was excised from agarose gel with the help of a clean scalpel.
2. The gel slice was weighed in a colourless tube and 3 volumes of QG buffer was added to 1 volume of gel.
3. The tube was incubated at 50°C for 10 minutes or until the gel dissolves. The tube was vortexed frequently to dissolve the gel.
4. After the gel got dissolved the colour of mixture was yellow, which indicated optimum Ph. 1 gel volume of isopropanol was added to the sample mixture and mixed properly.
5. A QIAquick spin column was placed in a provided 2 ml collection tube.
6. To bind DNA, the sample was applied to QIAquick spin column and centrifuged for 1 minute.
7. The flow through was discarded and QIAquick spin column was placed in the same collection tube.
8. 0.5ml of QG buffer was added to QIAquick spin column and centrifuged for 1 minute.
9. For washing o.75 ml of buffer PE was added to QIAquick spin column and centrifuged for 1 minute.
10. The flow through was discarded and QIAquick spin column was placed in the same collection tube and centrifuged for 1 minute at 17,900 x g (13,000 rpm).
11. QIAquick spin column was then placed in a clean 1.5 micro centrifuge tube.
12. To elute DNA, 50µl of EB buffer (10 mMTris-Cl, Ph-8.5) was added in the centre of QIAquick spin column and centrifuged for 1 minute.

The eluted DNA was stored at - 20°C until use.

3.5 Therapeutic studies

3.5.1 Surgical treatment of warts

Sedation: Cattle those bearing more than ten warts (i.e. those suffering from moderate grade infection) were sedated with 2% solution of Xylazine at a dose rate of 0.1 mg per kg body weight (0.25-0.5 ml/100kg bw) by intramuscular (I/M) injection and animals those suffered from mild grade infection (bearing medium to large sized warts, n<10) were administered 2% Xylocaine locally at the base of the warts or on the stalk. The animals were well restrained and tied before surgical excision of warts by one of the following regimes:

Regime-1: Surgical excision under local anaesthesia (2% Xylocaine) and thermocautery were done. Grossly forty animals (n = 40) those naturally had suffered from cutaneous papillomatosis (CCWT-35, and BCWT-5) were subjected to surgical intervention. Before surgical removal of the warts the animals were given 2% Xylocaine as local anesthesia infiltrated around the lesion, followed by cleaning with normal saline. Aseptically the warts were removed by surgical procedure. Excision of large sized warts was performed by sharp scalpel and haemorrhages were controlled by thermo-cautery. Surgical excision of warts was done under 2% Xylocaine at stalk. Twenty (n=20) warts/ papilloma infected cattle were treated by regime-1 where twenty papilloma (n=20) infected cattle were treated by thermo-cautery as post excision and another twenty wart (n=20) infected cattle were treated by potassium permanganate cauterization as post excision in Regime-2. The treated and control
animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

**Regime-2:** Surgical excision under sedation by 2% Xylazine I/M administration and potassium permanganate cauterization of twenty clinically affirmative cases of CWT (n=20) was adopted for studying the effect of surgical procedure on treating CWT.

**Regime-3:** Curetting of warts followed by trichloro-acetic acid application locally and S/C administration of Levamisole stat. to twenty (n = 20) warts infected animals for studying effectiveness of the treatment mode.

Curetting was done to remove overgrowth of warts by a scalpel till blood oozes to allow the reintroduction of virus into blood (Autogenous vaccine). Trichloro-acetic acid was applied to kill and to remove the rest wart cells. Levamisole was injected subcutaneously (S/C) at a dose rate of 1 ml/10 kg body weight as immune stimulant. Twenty (n = 20) papilloma infected cattle were treated by regime-3. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

Cattle in all treatment regimens were given standard mineral mixture and good plan of nutrition. Topical application of ayurvedic gel like Himax or Topicure was done on the skin wounds to prevent problems with secondary bacterial infection and myiasis.

**Cryotherapy:** Animals (n = 20) bearing multiple warts, were subjected to cryotherapy as a treatment mode for wart cure. Animals were restrained physically and lesions were cleaned with antiseptic solution. The part surrounding the lesion was protected using appropriate wooden guards to prevent cryo-damage to the adjacent tissues. The lesions were then frozen to -20 °C using liquid nitrogen cryo-system model-800-777 CRYO cryo-gun. The freezing temperature was monitored using a digital thermometer. The lesions were either subjected to cryotherapy by contact freezing or were spray frozen following double freeze thaw cycles for three consecutive sittings for three days apart. Effect of the therapy was monitored until complete clinical cure. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

3.6 Homeopathic remedy

**3.6.1. Animals and protocol Design**

One hundred twenty cows (age group 2-5 years) suffering from cutaneous papillomatosis from various cattle herds were included in the present investigation for studying the effectiveness of homeopathic medicine on CWT and TP. First of all clinically affected animals bearing multiple warts like cutaneous lesions were screened for the
investigation. Then, their size, shapes, location and numbers were precisely recorded and clinically examined in order to ensure that the animals were actually infected with papillomavirus. For this purpose, morphology and histo-pathological studies of the lesions were done with minimal manipulation. Thereafter ninety animals were randomly assigned to three equal groups (thirty animals in each group). Each animal of these three treatment groups was administered with 10-15 globules of sulphur of 200-power once orally for seven days (doses based on body weight and age) followed by administration of Thuza of different potency and route of administration whereas cattle for which saline was administered as placebo were used as negative controls (ten in each group).

3.6.2 Thuja mother tincture oral therapy

Group-1 (n = 30)

Animals of Group-1 consisted of 30 animals of which 28 were cattle (male-7 and female-21, CCWT-27 and CTP-1) and two were buffalo heifers suffering from cutaneous papillomatosis (BCWT-02). Among twenty eight cattle, CBJ HF were five in numbers (age group 2-3 years), HLX HF one (1.5 year), Gir HF four (average age-2.5 years), ND HF eight (average age-2.18 years), ND MC four (average age-10.5 months), ND FC two (10 months), Zebu bull calf one (1.5 year), ND BK two (average age-3.5 years), and Gir cow one in number. Average age of male cattle was 1.7 year. All thirty animals were treated with Thuja mother tincture. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

3.6.3 Thuja-200X oral therapy

Group-2 (n = 30)

Animals of Group-2 (n=30) consisted of 3 Murrah she buffaloes (average age-4.66 years) and 27 cattle, comprising 2- ND MC (average age-12.5 months), 3- ND BK (average age-3.66 years), 12-ND HF (average age-2.08 years), 8-CBJ cow (3-5 years), one CBJ FC (12 months) and 1-CBJ HF (1.5 Years) and all those were suffering from either cutaneous(CCWT-25 and BCWT-01) or teat papillomatosis (CTP-2 and BTP-2) and all thirty animals were given Thuja 200X @10-15 drops (according to the age of animals) twice daily respectively for 2 weeks. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period. Before and after treatment samples were collected for the histopathologic analysis and regression study.

3.6.4 Thuja-200X parenteral therapy

Group-3 (n = 30)
Animals of Group - 3 (n=30) consisted of 8-buffaloes (of which ND she buffalo were three and Murrah she buffalo were five in numbers, all suffering from BTP except one suffering from BCWT) and 22- cattle, comprising 2- ND HF(1.5-2 years), 3- ND FC(9-12 months), 2-ND MC (11 months each), 5-ND bull (3-3.5 years), 1-ND BK (3 years), 1 – Sahiwal cow (6 years), 3-CBJ cow(3-5 years) and 5-CBJ HF (2-3 Years) all suffering from either CCWT-20 or CTP-3. All thirty animals received a subcutaneous injection of Thuja 200x @2ml diluted with equal volume of distilled water once in a week for four weeks. Before and after treatment CWT samples were collected for the histopathologic analysis and regression study. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

3.7 Autogenous vaccination as therapeutic measure

3.7.1 Preparation and treatment with Autogenous vaccine

3.7.1.1 Regimen-1: (With formalinized autogenous vaccine prepared in sterile PBS)

Following the method of Hunt (1984) twenty (20) animals from various cattle herds with papilloma lesions on the skin elsewhere on animal body were selected to be used in the study. Their shape, size, location and numbers were precisely recorded. The lesions (comparatively old lesions) were partially removed surgically and individually from each animal by aseptic surgical procedure.

The papilloma lesions were thoroughly cleansed with lukewarm soap solution in order to remove the dung, soil and encrusted materials. The excised lesions were cut into small pieces by sterile scissors and rewashed by sterile PBS and then ground by using sterile sand and 10% suspension of the ground tissues were made by using sterile PBS. The suspension was centrifuged at 3000 rpm at 4° C for 30 minutes to remove the large particles. Supernatant fluid was collected in sterile bottle; formaline (0.4ml/100ml of supernatant) was added to the prepared vaccine and left for 24 hours at 4° C. The pH was adjusted to be 7.0 and then refrigerated until use.

To check the sterility of the vaccine, samples were inoculated in blood agar, nutrient agar and MacConkey agar at 37° C for 48 hour and inoculated in Sabarouds agar at 37 °C and 25° C for 3-7 days. Thus the autogenously prepared vaccines when proved sterile, the same animals with papilloma lesions were treated with their respective vaccine @10ml by subcutaneous route at the neck region at weekly interval for four occasions. Buffered normal saline was administered @ 5ml to five sick animals at the same route and interval that served as control. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.
3.7.1.2 Regimen-2 (With formalinised vaccine prepared in sterile normal saline solution)

The vaccine was prepared from sample collected from older wart growths under strict aseptic conditions, miniced, suspended in sterile normal saline solution, filtered through clean gauze to remove larger pieces of tissue materials, followed by Whatman filter paper no-1 and treated with formalin. Twenty animals (n=20) were treated with their respective vaccine as prepared with a dose of 5ml by subcutaneous route at the neck region at weekly interval for four occasions. Buffered normal saline was administered @ 5ml to five sick animals at the same route and interval that served as control. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

3.7.1.3 Regimen-3 (With formalinized autogenous vaccine, diluted in 50% glycerol-saline solution)

The preparation of the vaccine proceeded as per the methods of Pearson et al., (1958), Amstutz, (1978) and Wadhwa et al., (1995). Five grams of fresh active growth was removed surgically from individual animal under strict aseptic precautions. Tissues were cut into small pieces, homogenized in 50% glycerol-saline solution (30 ml/g of tissue), and the mixture was filtered through muslin cloth. Antibiotics (2, 00,000 of Procaine Penicillin and 250 mg of Dihydro-streptomycin sulphate) were added to prevent bacterial growth. Formalin 0.4 ml per 100 ml of filtrate was added to inactivate the virus and then kept in refrigerator for 24 hours. Twenty numbers of wart infected animals (n=20) were treated by autogenously prepared vaccine, administered S.C. twice at one week interval for four occasions. Buffered normal saline was administered @ 5ml to five sick animals at the same route and interval that served as control. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.

3.7.1.4 Regimen-4 (with autogenous vaccine prepared in 70% alcohol)

Sample from older growths from sick individual animals were resected under strict aseptic conditions, triturated the sample in 2ml of 70% alcohol to inactivate the pathogen. After trituration, filtered through sterile gauze followed by Whatman filter paper no-1 and mixed with 18 ml of sterile PBS and pH was adjusted to 7.0 and refrigerated until use. Twenty wart inflicted sick animals (n = 20) were thus administered in doses of 10 ml subcutaneously for four occasions at weekly interval. Buffered normal saline was administered @ 5ml to five sick animals at the same route and interval that served as control. The treated and control animals were examined every week and the characteristics of the warts were being compared with those of pre-treated period.
3.8 Auto-hemotherapy

3.8.1 Regime-1

For auto-hemotherapy 10-20 ml of venous blood was collected by jugular venepuncture and injected deep intramuscularly (I/M) @5ml/100kg body weight, in the same animal at weekly interval for four weeks. Self-blood therapy was carried out in 100 naturally infected animals (age group 1-5 years) irrespective of sex, breed, severity of infection and type of lesions. The treated and control animals were examined fortnightly and the characteristics of the warts were being compared with those of pre-treated period. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last administration.

3.8.2 Regime-2

Single dose of Levamisole orally along with self-blood therapy @5ml/100 kg body weight at weekly interval for four occasions were carried out in fifty naturally infected bovines (n = 50) irrespective of age, breed, sex, severity of infection and type of lesions. Animals were examined thoroughly for the regression or clinical cure at 15 days interval. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last application.

3.9 Immunomodulation

3.9.1 Levamisole oral therapy

Clinically, 50 cattle of different breeds, age, and gender bearing multiple cutaneous warts (CCWT cases -45 and CTP cases-5) received oral form of levamisole @ 2.5mg/kg body weight. Average age of the animals under this treatment group was 2.33 years. The animals under this mode of treatment were 4- NDHF (average age 2.25 years), 2-NDFC (average age-18 months), 3-NDMC (average age-8.66 months), 1-ND cow (3 –years), 3-NDBK (average age-5 years), 7-Gir HF (average age-19.71 months), 3-Gir FC(average age-10 months), two-RSHF (average age-16 months), one Sahiwal HF(2-years), one Sahiwal cow (3-years), 11-CBJ HF (average age-33-09 months), 4-CBJ cow (average age-4.75 years), 3-CBJ cow (average age-3.16 years), 2-CBJ MC (average age-10 months),1-HLX HF( 3 –years) and 1-HLX cow (4-years). Approximate total dose was calculated according to the body weight of the animal. Another 50 animals was kept as control and received no treatment. Treatment was applied on 1, 3, 5, 7, 16th days as per the method of Cihan et al., (2004). The treated and control animals were examined at 15 days interval and the characteristics of the warts were being compared with those of pre-treated period. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last administration.
3.9.2 Levamisole parenteral therapy

Therapeutic studies were made on 50 (n = 50) naturally infected clinical cases of both cutaneous and teat papillomatosis (CCWT, n = 45 and CTP, n = 05) in four ND MC (average age-11.25 months), one ND FC (9 months), two CBJ FC (average age-19 months), two Gir FC (average age-10 months), five NDHF (average age-2.2 years), four Gir HF (2.12 years), two Sahiwal HF (average age-3 years), fourteen CBJ HF (average age-2.25 years), one Gir bull (3 years), seven ND BK (average age-3.14 years), one ND cow (4 years), five CBJ cows (average age-3.4 years), one HLX cow (4 years) and one ND bull (2 years). Average age of the animals under this treatment group was 2.32 years. All fifty animals received injectable form of levamisole @ 2.5mg/kg body weight by subcutaneous route. Approximate total dose was calculated according to the body weight of the animal. Another 50 animals were kept as control and received no treatment. Treatment was applied on 1, 3, 5, 7, 16th days. Clinical efficiency of the treatment was monitored for three months. The treated and control animals were examined at 15 days interval and the characteristics of the warts were being compared with those of pre-treated period. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last administration.

3.10 Ivermectin therapy

All together one hundred eighty clinical cases of bovine cutaneous papillomatosis from different affected herds from various districts of Bengal was included in this study. Effect of Ivermectin per os (oral therapy), single S/C administration and double shot S/C administration of Ivermectin (parenteral therapy) was studied. All three different modes of treatment were carried out in thirty animals in each regimen keeping thirty clinically diagnosed naturally infected cases of CCWT as control. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last administration.

3.10.1 Ivermectin oral therapy

Thirty naturally infected clinical cases of cutaneous papillomatosis (CCWT-28 and CTP-2) of cattle of different breed, age and gender were randomly selected and were included in therapeutic study. Ivermectin oral therapy was carried out in one Gir MC (10 months), one Gir HF (3 years), one HLX cow, one ND FC (8 months), seven ND MC (10 months -1 year), seven ND HF (2-3.5 years), one ND cow (4 years), two CBJ cow (3 years) and nine CBJ HF (2-3 years). These animals had wart like lesions on cutaneous surface having different growth pattern, varying size and shape. Some of the papillomas were pedunculated and some were devoid of stalk. Oral preparation of Ivermectin was given @0.2mg/kg bw for two occasions at
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15 days interval to all thirty animals and animals were observed at 15-day intervals for 90 days. The treated and control animals were examined at 15 days interval and the characteristics of the warts were being compared with those of pre-treated period.

3.10.2 Ivermectin parenteral therapy (Single dose S/C administration)

Clinically, 30 cattle of different breeds, age, and gender and body weight, bearing multiple cutaneous warts received single shot injectable form of Ivermectin @0.2mg/kg body weight subcutaneously. Clinical trial was carried out in three RS HF (average age - 1.83 years), five Gir HF (average age - 2 years), seven CBJ HF (average age - 2 years), one ND HF (2 years), two CBJ MC (average age - 10.5 months), four ND MC (average age - 9.25 months), one CBJ FC (10 months), two Gir FC (average age 10 months), three ND FC (average age - 10.33 months) and two ND BK (average age 4 years). These animals had cauliflower-like masses and growths of varying morphology, shapes and sizes above the skin surface at different anatomical locations. Approximate total dose was calculated according to the body weight of the animal. Another 30 animals was kept as control and received no treatment.

3.10.3 Ivermectin parenteral therapy (Double shot S/C administration)

Clinically, 22 cattle and 8 buffaloes of different breed, age and gender bearing either cutaneous or teat lesions, clinically diagnosed as warts or papilloma were included in the present mode of clinical trial. Two Gir FC (average age 11.5 months), one Gir HF (3 years), one RS HF (16 months), three ND HF (average age - 2.16 years), two ND BK (average age - 3.5 years), two CBJ MC (average age - 11 months), four CBJ FC (average age 11 months), five CBJ HF (average age - 2.2 years), one CBJ cow (5 years), one HLX HF (3 years), three ND BF (average age 4 years), one CB MB BF (3 years) and four MB BF (average age - 4 years) were included in the study and received treatment. Among bubaline papillomatosis cases, BCWT cases were two in numbers and BTP cases were five in numbers. And in case of bovine papillomatosis, CCWT cases were twenty one in number and CTP was one. All thirty animals received two shots of Ivermectin injection at 15 days interval @0.2mg/kg body weight subcutaneously. Another 30 animals was kept as control and received no such treatment. Control animals received only 25 ml of NSS subcutaneously at the neck region. These animals had cauliflower-like masses and growths of varying morphology, shapes and sizes above the skin surface at different anatomical locations. Approximate total dose was calculated according to the body weight of the animal. Animals were observed at 15-day intervals for three months.

3.11 Therapy with Antimony Preparation (Lithium antimonium thiomalate)

Injectable form of Antimony compound (namely Injection Anthiomaline ® of Novartis, was used in the present study. Control group of animals did not receive any treatment. Only normal saline was administered subcutaneously.
3.11.1 Anthiomaline therapy in clinically affected case of teat papillomatosis

Therapeutic studies were made on ten (n=10) spontaneous cases of teat papillomatosis (in six crossbred Jersey and four crossbred Holstein Friesian milch cows) in dairy herds under Kolkata Pinjrapole Society, keeping another ten such naturally infected clinical cases as control too. No cutaneous lesions other than teat or udder warts bearing animals were included in the present investigation. Detailed clinical examination gross morphological studies of the clinically affected ten animals in treatment group revealed that warts were pedunculated, filiform with rough external surface in six cross Jersey cows, while in remaining 4 animals warts were of large frond and sessile type. There was progressive development of multiple irregular hard projections like warts over the normal udder and teats both (in six cows) and over teats only in four cows since last few months.

All ten animals in the treatment group were treated with (Lithium antimony thiomalate) (i.e. Anthiomaline) @ 10-15 ml deep I/M, depending on the body weight of the animals. Five injections on alternative days (1, 3, 5, 7 and 9th days) and injection Chlor-pheniramine maleate @ 10 ml on 1, 3, 5, 7 and 9th days including messaging of warts with honey and vinegar in equal proportion in each milking was carried out. Clinical efficacy was monitored for four weeks. A follow up treatment would be resorted again after four weeks in non-responding cases.

3.11.2 Anthiomaline therapy in cutaneous warts

3.11.2.1 Anthiomaline therapy (alone) in cutaneous warts

Parenteral therapy with Lithium antimony thiomalate (Injection Anthiomaline) was carried out in all forty five animals (CCWT, n = 43 and BCWT, n = 01 and BTP, n = 1) those had suffered from spontaneous cutaneous papillomatosis. Animals (CBJHF-19, NDHF-11, NDMC-2, NDFC-2, CBJ BK-1, CBJMC-2, HLXMC-1, HLXFC-1, ND BK-2, MBHF-1, MB she buffalo-1) were treated with (Lithium antimony thiomalate)(i.e. Anthiomaline)@ 10-15 ml deep I/M, depending on the body weight of the animals. Accordingly five injections of Anthiomaline on alternative days (1, 3, 5, 7 and 9th days) and injection Chlor-pheniramine maleate @ 10 ml on 1, 3, 5, 7 and 9th days were administered by I/M route. Control group of animals did not receive any treatment. Only normal saline was administered subcutaneously The treated and control animals were examined at 15 days interval and the characteristics of the warts were being compared with those of pre-treated period. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last administration.
3.11.2.2 Combined therapy with oral Ivermectin and Parenteral Anthiomaline therapy in cutaneous warts

Parenteral therapy with Lithium antimony thiomalate (Injection Anthiomaline) along with single dose Ivermectin per oral route (oral formulation of Ivermectin as per the body weight of the animals @ 0.2mg/kg bw) was tried for treating cutaneous papillomatosis in forty five clinical cases of papillomatosis. Therapeutic studies were made on 45 (n = 45) naturally infected clinical cases of cutaneous papillomatosis (CTP-6, CCWT-38 and BCWT-01) in eight ND MC (10.1 months average age), one ND cow (3 years), three NDFC (average age 10 months), two Gir FC (average age 11.5 months), seven CBJ cow (average age3.8 years), one CBJ MC (11 months), six CBJ FC (average age10.1 months),five NDHF (average age 2.15 years), two Gir HF (average age 2 years), two CBJ BK (average age3.5 years), two ND BK (average age2.8 years), five CBJ HF (average age2.4 years) and one MB she buffalo (4 years). All forty five animals received a single dose of Ivermectin as per the body weight of the individual animal @0.2 mg/kg orally. All forty five animals in the treatment group were then treated with Lithium antimony thiomalate (i.e. Anthiomaline) @ 10-15 ml deep I/M, depending on the body weight of the animals. Five injections on alternative days (1, 3, 5, 7 and 9th days) and injection Chlor-pheniramine maleate @ 10 ml on 1, 3, 5, 7 and 9th days were administered intramuscularly and animals were observed at 15-day intervals for 75 days. The treated and control animals were examined at 15 days interval and the characteristics of the warts were being compared with those of pre-treated period. Before and after therapy, wart samples were collected for the histopathologic analysis and epithelial regression study. The animals were observed in a 90 days period after the last administration.

3.11.2.3 Combined therapy with single S/C administration of Ivermectin and Parenteral Anthiomaline in cutaneous warts

Parenteral therapy with Lithium antimony thiomalate (Injection Anthiomaline) along with single dose S/C administration of Injectable form of Ivermectin was tried for treating cutaneous papillomatosis. Therapeutic studies were made on 45 (n = 45) naturally infected clinical cases of cutaneous papillomatosis (CCWT, n = 44 and BCWT, n = 01) in one ND MC (11 months), one CBJ MC (14 months), one CBJ FC (10 months), five NDHF (2-2.5 years), five Gir HF (1.5-3 years), two RS HF (2.5-3 years), twenty CBJ HF (1.5-3.5 years), one Sahiwal HF (3 years), three ND BK (3-5 years), two hill cows (4 years), three CBJ cows (4-5 years) and one MB she buffalo (4 years). All forty five animals received a single dose injectable form of Ivermectin as per the body weight of the individual animal @0.2 mg/kg subcutaneously. All forty five animals in the treatment group were then treated with Lithium antimony thiomalate (i.e. Anthiomaline) @ 10-15 ml deep I/M, depending on the body weight of the animals. Five injections on alternative days (1, 3, 5, 7 and 9th days) and injection Chlor-
pheniramine maleate @ 10 ml on 1, 3, 5, 7 and 9th days were administered intramuscularly and animals were observed at 15-day intervals for 75 days. The treated and control animals were examined at 15 days interval and the characteristics of the warts were being compared with those of pre-treated period. Before and after therapy, wart samples were collected for the histopathologic analysis and warts regression study. The animals were observed in a 75 days period after the last administration.