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Molecular diagnosis of bovine papillomatosis and a successful management with autogenous vaccine

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Abstract
Bovine papillomatosis is caused by papilloma viruses. Thirty six cases of bovine papillomatosis were brought to the clinics, Department of Surgery and Radiology, C.V.Sc, SVVU, Tirupati. On clinical observations of the wart lesions, it was diagnosed as bovine papillomas. Wart samples were collected aseptically and processed for Molecular diagnosis. All the thirty six wart samples were subjected for for homogenization under sterile conditions with tissue lyser.

All the thirty six wart samples were subjected for homogenization under sterile conditions with tissue lyser. DNA was extracted from tissue homogenates and subjected for molecular diagnosis with PCR targeting L1 gene. Out of 36 samples, five (5) were positive for BPV Type-1, eight for positive for BPV type -2 and eleven were positive for both BPV-1 & BPV-2 and remaining twelve samples were positive for BPV Type-5 with species specific primers. Later as a therapeutic measure, an autogenous vaccine was prepared. The wart samples collected aseptically from all the affected animals and adjuvanted with aluminium hydroxide. After sterility checkup the vaccine was administered for affected animals subcutaneously to cows @10ml and heifer calves@5ml on 0th day and then subsequently 10 days interval for 5 doses. The regression of the warts was started by three weeks of post vaccination and complete regression was appreciated by 6th week. The study represents the presence of the bovine papilloma virus type 5 on molecular diagnosis and successful management of bovine papilloma with bovine specific autogenous vaccine.

Keywords: Papillomatosis, wart lesions, molecular diagnosis, autogenous vaccine-regression

Introduction
Papilloma viruses are a diverse group of small, non-enveloped, Circular double stranded DNA viruses that infect various animal species and humans. (Antonsson and Hansson, 2002) [1]. The virus normally infects epithelial cells causing benign hyper proliferative lesions (warts, papillomas and fibro papillomas) which can progress to cancer (Campo, 2006) [9]. Currently, 15 types of BPV types (BPV -1 to 15) are described (Munday *et al*. 2015) and classified in four genera. Delta papilloma virus (BPV1,2,13 and 14) epsilon papilloma virus (BPV-5&8), Xiapapilloma virus (BPV-3,4,6,9,10,11,12 &15) and Dyoxipapilloma virus (BPV-7) (Melo *et al*. 2014; Grindattoo *et al*. 2015; Munday *et al*. 2015; Silva *et al*. 2016). Delta and epsilon papilloma virus are associated with both papilloma and fibropapillomas, while xipapilloma virus only to squamous papillomas (Tan *et al*. 2012b; Araldi, 2015 and Aradi *et al*. 2015b) [2].

The infection can result in significant economic losses in animal husbandry due to mastitis, lower milk and meat yields and reduced hide quality (Camp 2002 & 2006; Jeilnek & Tachezy 2005) [9, 14].

Diagnosis of infection is based on clinical symptoms, histopathological examination of tumor growth, Immuno histochemistry and the use of electron microscopy (Turk *et al*., 2005) [33]. As virus entry result in an asymptomatic and latent infections. The conventional methods of histopathology, immune histochemistry are laborious and time consuming. Polymerase Chain reaction (PCR) remains an important tool for early diagnostic purposes. Particularly in asymptomatic carriers in latent infections either in epithelial tissues or non epithelial tissues and body fluids such as blood, milk, colostrum, urine, semen, uterine discharges, ovary, oocysts and placenta etc. (Lindsey CJ *et al.* 2009) [19]. Further, there is no efficient invitro culture system for growing the virus and bio-typing of the prevalent virus types is not possible by serology. Hence the present study revealed the molecular diagnosis of Bovine Papillomas and the use of autogenous vaccine in cattle as a successful managemental practice.

Materials and Methods

Sample collection

Thirty six animals, cross bred heifer (22) and adult cows (14) were brought to the clinics, Dept. of Veterinary surgery and Radiology, College of Veterinary Science, SVVU, Tirupati with a history of papillomas during the years 2017 & 18. On clinical examination small to big wart like lesions around the eyes, ears, nose, neck, shoulder, abdomen and udder varying in size from 0.5 to 50 mm in diameter was observed. The lesions were characteristic and varied from flat to pedunculated. The animals were apparently healthy on clinical observation and the cases were suspected as bovine papillomatosis. Later wart samples were collected on ice from all the thirty six animals, processed for molecular diagnosis and autogenous vaccine preparation.

Molecular Diagnosis

PCR - DNA Extraction

The collected wart samples (36.No's) were washed thoroughly with sterile PBS. Then cut into small pieces. Later subjected for homogenation with tissue lyser. DNA was extracted from all the thirty six samples with DNA extraction kit from Tissues (Qiagen) as per the manufactures protocol. Polymerase chain reaction was performed according to the method of Pawan Kumar *et al*, 2013 [29]. PCR was performed targeting the L1 gene of BPV 1, 2, 5 and 10 using type specific primers (table.1 & 2)

Table 1: Primers used in the study for amplification of BPV Types in AP

Primers	Primer sequence	Amplicon size
BPV-1- For	GGAGCGCCTGCTAACTATAGGA	301bp
BPV-1- Rev	ATCTGTTGTTTGGGTGGTGAC	
BPV-2- For	GTTATACCACCCAAAGAAGACCC	164bp
BPV-2- Rev	CTGGTTGCAACAGCTCTCTTTCT	
BPV-5- For	ACTGGCTCTACCAAGCTCAAGG	266bp
BPV-5- Rev	GACAGAAGGGTTAACGGTCTGCA	
BPV-10- For	TGCATTCAATAGGCTTGCAGATGC	422bp
BPV-10- Rev	CACCTCGAGACCACAAATGC	

Table 2: PCR cyclic conditions

Initial Denaturation	94°C for 3 min	
Denaturation	94ºC for 40 sec	Repeat 35 cycles
Annealing	52º C for 40 sec(BPV- 1 & 2) 62º C for 40 sec (BPV- 5) 60º C for 40 sec (BPV- 10)	
Extension	72º C for 50 sec	
Final Extension	72º C for 10 min	

PCR Reaction Mixture

PCR Master Mix - 12.5µl
BPV - Forward Primer - 1 µl
BPV - Reverse Primer - 1 µl
Template DNA - 2 µl
Nuclease free water - 8.5 µl

Analysis of PCR Products

Amplified DNA fragments were visualized and documented by Gel documentation system (Bio-Rad) in 1.5% agarose containing ethidium bromide (0.5g/ml) as per standard procedures.

Autogenous vaccine Preparation

Wart lesions were collected from all the thirty six animals, i.e. adult cows (14) and heifer calves (22) aseptically in PBS on ice until processing according to the method described by Hunt (1984) [13]. The process was carried out under sterile conditions. The collected wart tissues were washed thoroughly with sterile PBS and cut into small pieces with sterile scissors and homogenated with sterile PBS. Then the suspension was centrifuged at 3000rpm for 30 minutes at 4^0C to remove the coarser particles. Supernatant was taken and formalin was added at a concentration of 0.5% to inactivate the virus. Vaccine thus prepared was adjuvanted with equal volumes of aluminium hydroxide and left for 24 hrs at 4^0C.

As a sterility checkup, the adjuvanted vaccines were inoculated on blood agar, nutrient agar and Maconkeys agar for aerobic bacteria and into thioglycolate medium for anaerobic bacteria and incubated at 37^0C for 48hrs.

Similarly for fungal checkup the vaccine samples were inoculated on Sabourauds dextrose agar media and all the inoculated plates were kept in duplicates, one at 37^0C and another at room temperature for 3-7 days. After the sterility checkup the same animals with papilloma warts were administered with the vaccines thus prepared.

Vaccine Dose and administration

After sterility checkup the affected adult (9 cows) animals were administered with 10 ml of vaccine dose subcutaneously at the shoulder region and the heifer calves (3.Nos) with 5ml dose subcutaneously with similar manner. The second dose was given 10 days after the first dose. Then subsequently third, fourth and fifth doses were given in similar manner at 10 days interval and observed for regression of warts.

Results

A total of 36 cases of Bovine Papilloma affected animals comprising of adult animals (14. Nos) and heifer calves (22.No's) brought to the clinics were studied. Initially all the cases were diagnosed as papillomas based on clinical presentation and wart like lesions on different parts of the body on head, neck (fig.1 to 4) shoulder. Grossly the warts were of varying sizes with rough, irregular shape, cauliflower like growths.

Polymerase Chain Reaction

Out of thirty six (36) samples collected from affected animals, five samples showed positive to BPV type -1 yielding 301bp, eight samples showed positivity to BPV type-2 yielding 166bp and 11 samples were positive for both BPV-1 and BPV-2. Remaining twelve samples yielded a amplicon size of 266bp corresponding to the expected L1 gene fragment of BPV 5. (Fig.5 &6).

A Successful Management of Bovine Papillomatosis with Autogenous Vaccine in Cattle

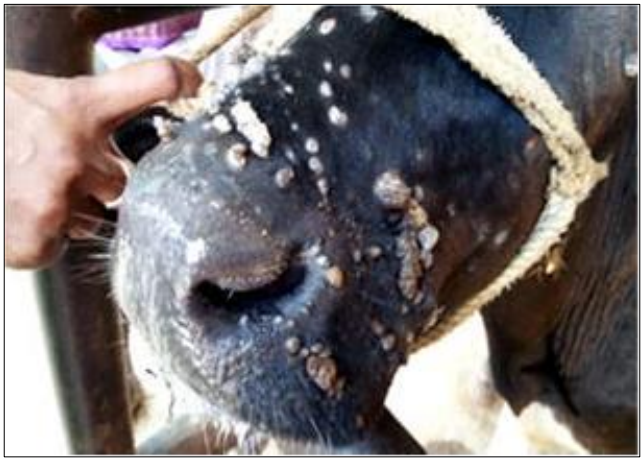


Fig 1: Papilloma case before autovaccination (Heifer calf)

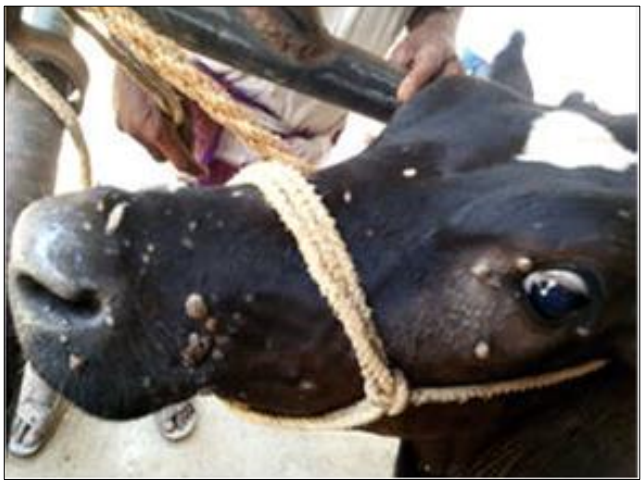


Fig 2: After autovaccination (heifer calf)

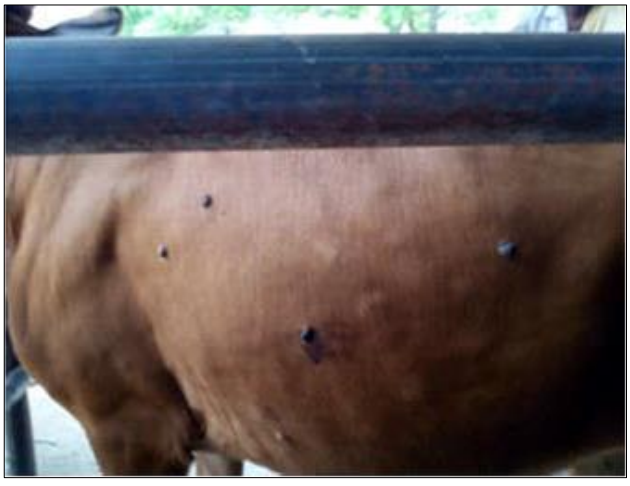


Fig 3: Papilloma case before autovaccination (adult cow)

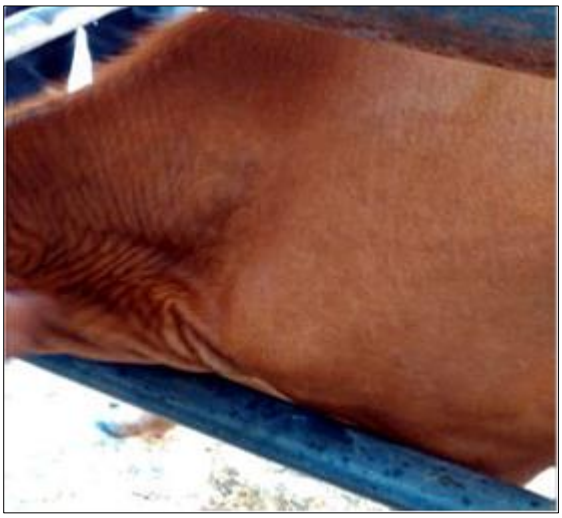
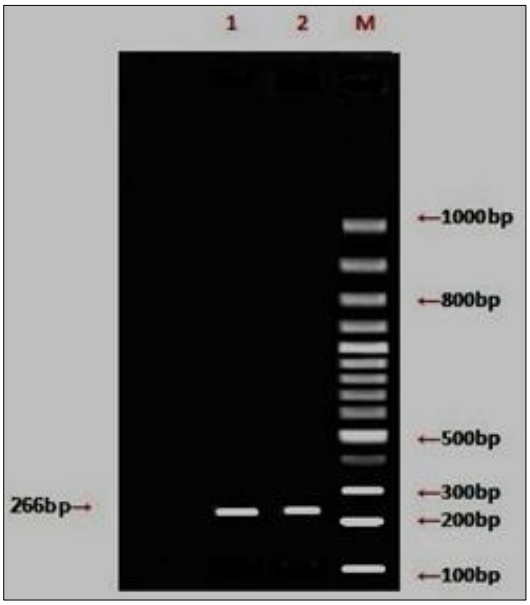
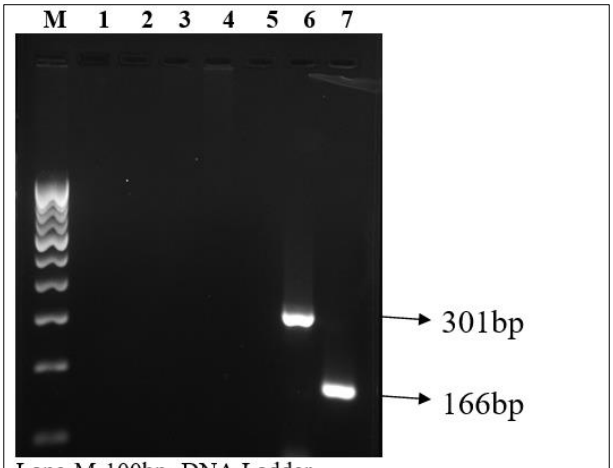


Fig 4: After auto vaccination (adult cow)



Lane- 1 & 2-266bp amplified PCR products specific for BPV-5.
Lane-M-100bp DNA Marker

Fig 1-4: Molecular Detection (PCR) of Bovine Papilloma virus type -5



Lane-M-100bp -DNA Ladder.
Lane- 6- 310bp amplified PCR product specific for BPV-1.
Lane-7- 166bp amplified PCR product specific for BPV-2.

Fig 5: Molecular Detection (PCR) of BPV type -1 & 2

Autogenous vaccine

Administration of autogenous vaccine caused sloughing of the warts from the affected areas. The papilloma lesions were started regression after third dose of post vaccination of animals and the complete regression was observed by sixth week of post vaccination in less severe cases and by seventh week in severely affected cows.

Discussion

Bovine Papillomatosis is a common viral disease of the skin, manifested as benign tumour or warts, caused by bovine papilloma virus (Oslon, 1993). Papilloma virus may affect all ages of cattle. Diagnosis of cutaneous papillomatosis, which is commonly observed in cattle, is based on clinical symptoms, histopathological findings and the use of electron microscope Turk *et al*. 2005 [33].

During the study cutaneous papillomatosis was diagnosed based on clinical symptoms and lesions all over the body and confirmed by identifying nucleic acid with PCR using type specific primers for amplification of L1 gene. During the study BPV-1 was detected in five samples and is in agreement with earlier reports of Freitas *et al*. 2014; Jelinek *et al*. 2005 and Kumar *et al* 2013 [29]. BPV-2 was detected in eight samples during the study. Several workers Claus *et al*. 2009, Silvestre, *et al*. 2009 [31] & Singh *et al*. 2010 [32] reported the presence of BPV-2. Twelve samples revealed the presence of BPV-type-5 during the study. Previously Lindholm *et al*. 1984 [17], Bloch *et al*. 1994 [4] and Jangir *et al*. 2017 [15] reported the prevalence of BPV-5 type. Both BPV-1 and BPV-2 types were reported in eleven samples in the present study. Similar findings were observed by Lancaster and Olson 1978; Leishangthem *et al*. 2008 [33]; Pangty *et al*. 2010 [27]; Pathania *et al*. 2011 [28]; Carvalho *et al*. 2012 [12]; Silva *et al*. 2012 [12] and Pawan Kumar *et al*. 2013 [29]. Hence, the etiological agents for cutaneous fibropapillomas were found to be BPV-1, BPV-2 and BPV-5.

The successful treatment of papillomatosis has been a great challenge for field practioners. Commercially no vaccines are available in India. Different methods have been used to treat bovine papillomas. Surgical intervention may not be possible if a large area is involved and effective medicines for wart are not available presently. Though bovine papillomatosis is a self limiting disease, autogenous vaccine is alternative therapeutic remedy for Bovine Papillomas.

Hence, in the present study, an autogenous vaccine prepared from warts of affected animals was used as a therapeutic measure for successful treatment of papillomatosis. The successful recovery of papillomas was observed by sixth week in less severe cases and by seventh week in severe cases with type specific autogenous vaccine. Similar findings were reported by Hamed *et al.* 2012. But Ndarathi and Mbuthia 1994 made an observation that a autogenous vaccine prepared from a single wart sample from one animal fail to cure other infected animals and probably it may be due to difference in the etiological agents of BPV types.

Further, Campo (1999 & 1997a, 1997b) [7, 5, 6] reported that Bovine Papilloma viruses 1,2 & 5 cause fibropapillomas and the vaccine prepared using all the three virus types results in successful treatment of Bovine Papillomas. During the study most of the animals affected were of younger ones within two years age group and this is in agreement with Radostitis *et al.* 1994 [30] but the author also reported that older animals were resistant to the infection and it could be due to immunity acquired from apparent and in apparent infections.

Nenad Turk *et al*. 2005 [33] reported the use of parammunity inducer (Baypanum, Byer pharma, Germany) along with autogenous vaccine shown a beneficial effect in treatment of bovine papillamatosis. However, no parammunity inducer has been used during the study.

Thiaya *et al*. 2009 also suggested that the wart samples should be collected from all infected ones and with those material common vaccine can be prepared for successful treatment of Bovine Papillomas. Further owners of the all affected animals were advised for good managemental practices.

Conclusion

In conclusion, BPV-1; BPV-2, mixed co-infection of BPV-1& BPV-2 & BPV-5 were identified as a cause of bovine cutaneous fibropapillomatosis on molecular techniques. However, further study is required region wise to understand the epidemiological, clinical and immunological features of cutaneous fibropapillomatosis and to find out most prevalent BPV types causing cutaneous warts in Bovines. This will help in designing future vaccine in therapeutic control of cutaneous fibropapillomas in Bovines.

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Conflict of interest

The authors declare that they have no conflict of interest.

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