

# Novel Molecular Detection of BPV-10 and BPV-11 Genotypes in Al- Anbar, and Baghdad Provinces

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## Abstract

In this study, the prevalence of diverse bovine papilloma viruses in cutaneous lesions was investigated in Iraqi cows. Fourteen specimens were taken from udder growths of Iraqi dairy cows, which demonstrated rice grain-like and small, raised flesh-like appearance. DNA was extracted from the teat wart samples, then was amplified by the polymerase chain reaction (PCR) using primers against BPV-10 (403 bp) and BPV-11 (560 bp). PCR results revealed mixed infections of BPV-10 and BPV -11 in four samples, while the remaining ten samples were infected with BPV-10 only. These findings demonstrate the presence of bovine papillomaviruses 10 and 11 in rice grain-like cutaneous lesions on the teats of cows and multiple elevated growths on udder. The detection and report of the BPV-10 and BPV -11 genotypes in the current study is for first time in Iraq, which are known in the Middle East region, are crucial for disease control measurements.

**Keywords:** BPV-10, BPV-11, Genotypes, Molecular detection, PCR

## Introduction

Cutaneous warts are spread between cattle and originate from the bovine papilloma (BPV) <sup>1</sup>, of which there are multiple strains. The lesion can generally be described as benign, yet may turn malignant <sup>2</sup>. BPVs exhibit tropism for mesenchymal and mucosal tissue as well as squamous epithelium <sup>3-6</sup>. Studies have identified fourteen virus genotypes that have been grouped into four genera: Dyoxipapillomavirus, Deltapapillomavirus, Epsilonpapillomavirus, and Xipapillomavirus <sup>7-10</sup>. BPV-10 and BPV-11 belong to the Xipapillomavirus family, which contains another type of BPV like viruses <sup>5</sup>, which are associated with the production of true papillomas <sup>11</sup>.

BPV is naked, icosahedral, and possesses double-stranded circular DNA that is 8000 base pairs (bp) containing 5 or 6 open reading frames (ORFs) that

are expressed early during infection. Besides two ORFs that are expressed late during infection, the genetic material is organized into three regions: early domain, a long control domain, and a late domain, within which diverse necessary and vital proteins are encoded <sup>12</sup>. With cofactors such as environmental carcinogens present, BPV-induced benign lesions degenerate naturally, but may also develop into cancer <sup>4</sup>. Clinical, histopathological, and immunohistochemical examinations are all usually used in BPV diagnosis <sup>13</sup>. Molecular characterization of bovine papillomavirus in Iraqi cattle showed the widespread presence of this oncogenic virus and the contribution it has to the development of skin tumors. BPV1, 2, and 13 affect dairy and beef cattle and induce remarkable commercial losses <sup>14,15</sup>. This study aims to investigate and identify the etiology of teat and udder warts of Iraqi dairy cattle using molecular and histopathological approaches.

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**Approaches and protocols**

**Biopsies**

Fourteen teat cutaneous papilloma samples were taken from bovine farms in the Al-Anbar and Baghdad province, including some brought to private clinics between August 2015 to May 2017. The samples that were collected demonstrated a variation in diameter range, about one to three centimeters, and collected from various portions of the

teat and udder (Figure 1). For samples that would undergo molecular biology analysis, deep freezing was undertaken, whilst 10% neutral buffered formalin were used to fix samples undergoing histopathological analysis.

**Histological study:**

Teat and udder specimens were fixed and processed according to routine protocols, as described previously (16). After fixation in 10% NBF, tissue fragments were processed with xylene, dehydrated using ascending concentrations of ethanol (70 to 100%), and set in paraffin, after which they were incised to 5-m pieces, mounted into the slides, and stained by hematoxylin and

eosin (H&E) and observed by veterinary pathologist under light microscopic and photographed.

**DNA extraction:**

Automated DNA extraction system was used to extract genomic DNA (Magesia, Anatolia Geneworks, Turkey) was utilized in this analysis following the manufacturer’s instructions. The -80 degree freeze samples were processed via an automated Magesia DNA Extraction machine.

**BPV-10 and BPV-11Primer designing**

Primers were designed by aligning the viral genome by the ApE program ([http://biologylabs .utah.edu/Jorgensen/wayned/apE](http://biologylabs.utah.edu/Jorgensen/wayned/apE)): and NCBI Primer-BLAST. These specific primers for genotyping were made by selecting conserved regions for each genotype specifically designed for BPV-10 and BPV-11. Primer sequences are shown in Table 1. The primers were designed to target conserved regions for alignment of BPV-10 (BPV-10, AB331651, and BPV-10 isolate KF017607, MYP55) and BPV-11 complete genome (BPV-11 DNA, AB543507) by the use of the ApE program. The designed primers were tested for inclusivity and exclusivity.

**Table 1 Primers for BPV-10 and BPV-11**

	<b>BPV-10 (403bp amplicon)</b>	<b>BPV-11 (560 bp amplicon)</b>
forward	5-GGACAAATGGCACAGGGGTA-3	5- GCCTGCAACTAGGGTCTCTG -3
reverse	5- CTGTTGTACGACGCTGGAGT -3	5- TAGATCGCGATGACGACTGC -3

**PCR Amplification**

PCR was done utilized a Thermal Cycler (SureCycler 8800, Agilent Technologies, USA) with each individual reaction prepared to a final volume of 25-L containing the following:

100–250ng DNA,
2mM MgCl <sub>2</sub> ,
1.25-L primers
(0.5-M), and 12.5-L 1X Abm HotStart Ready-mix (Applied Biological materials, Canada).

The PCR assay procedure included the following

Starting denaturation ..... 94°C for 180s.	
Denaturation ..... 94°C for 20s, Annealing ..... 44°C for 15s, Extension ..... 72°C for 30s.	35 Cycle
Extension, final step .....at 72°C for 10 minutes /kb	

BPV DNA PCR products were identified by a 1.5% agarose gel containing ethidium bromide electrophoresis assay. Gels were put in TBE buffer and ran at a fixed voltage of 100V for thirty-five minutes and visualized via a gel Documentation System (VISION, Scie-Plas, UK). Clinically healthy slaughtered cattle DNA extracted from skin tissues were used as negative control.

### Results

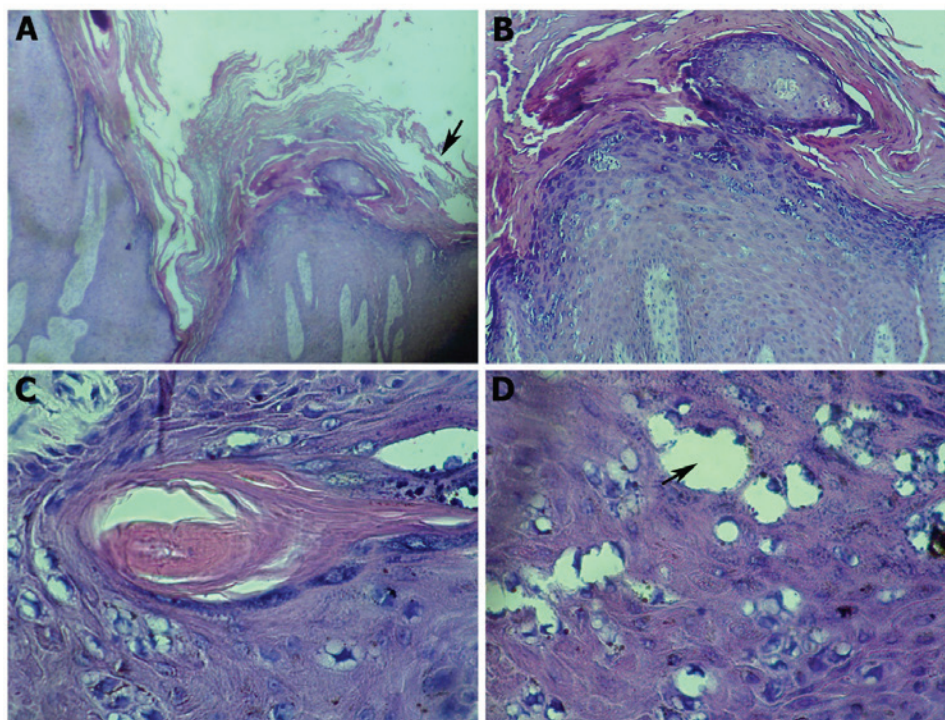
Clinically diagnosed teat wart cases in the dairy cows, demonstrated a macroscopic appearance as rice grain-like, small, flesh-like growths.

These were generally diffused and enriched with vascularity (see Figure 1A and B). Gross examination revealed that skin warts were exophytic. The histopathological study describes the tumors as cutaneous papillomas (benign squamous cells). Microscopically, all tumor growths showed comparable histological characteristics; according to different degrees of parakeratosis or hyperkeratosis with the presence of papillary-like projections of the squamous epithelium (Figure 2A and B).

In all tumor specimens assessed, most keratinocytes had a clear halo surrounding the nuclei, while others exhibited a pyknotic nucleus that is described as koilocytes (Figure 2C). Presence of koilocytes that have severely condensed pyknotic nucleus with basophilic staining, in addition to multiple degenerated keratinocytes (Figure 2D).

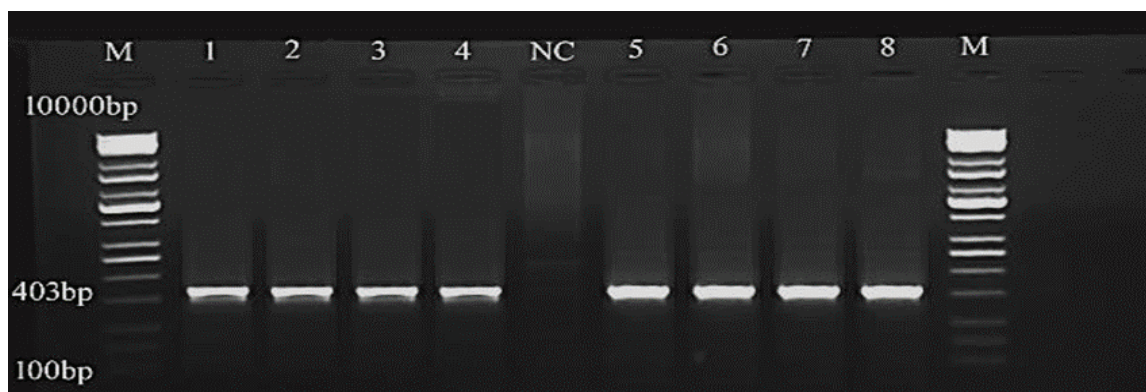


**Figure (1) A) cow suffering from teat papilloma. B) Cow suffering from multiple udder Papilloma**



**Figure-2, Histopathological characterization of teat and udder warts in a cow. A) Presence of papillary-like projections of the squamous epithelium. Stratum corneum showed a hyperkeratosis (black arrow), H&E, 40x. B) The proliferation of the squamous epithelium that has koilocytes can be observed H&E 100x. C) Hyperkeratosis and presence of clear halo surrounding the nuclei of most keratinocytes H&E 400x. D) Presence of koilocytes that have severely condensed pyknotic nucleus with basophilic-staining. In addition to multiple degenerated keratinocytes (black arrow). (H&E, 400x).**

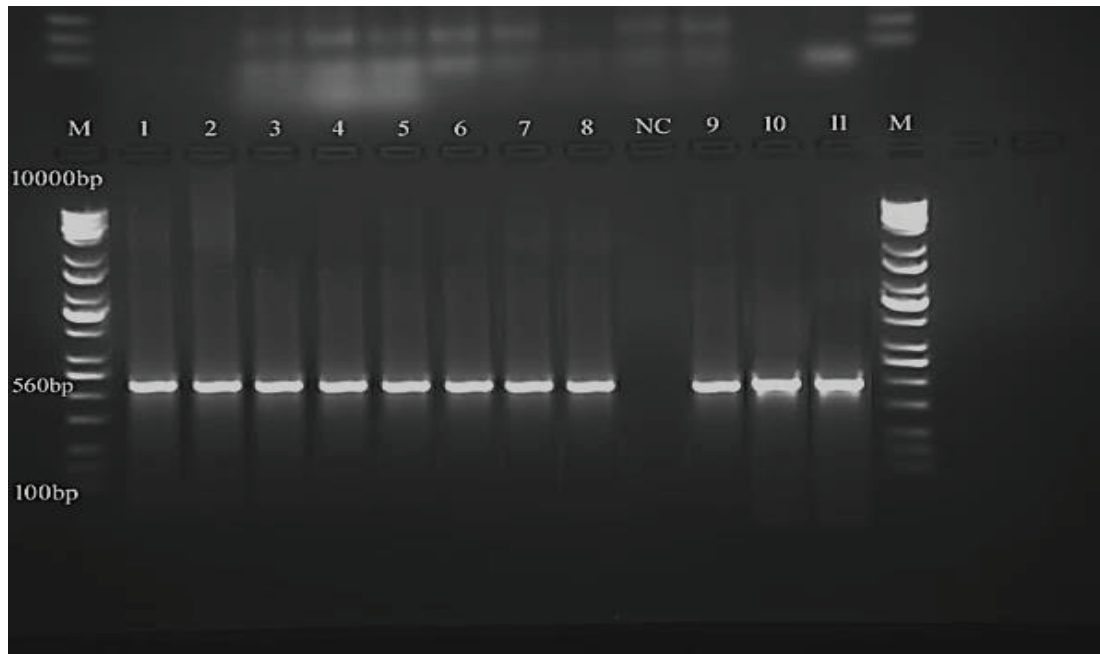
Bovine papillomavirus type 10 (BPV-10) DNA was discovered exclusively in ten (71.42%) of the fourteen teat and udder wart samples (Figure 3, Table 1), whilst four samples (28.58%) showed mixed infections with BPV-10 and BPV-11 (Figure 4, Table 1) which belong to Xipapillomavirus family<sup>(8)</sup>. These BPV-10 and BPV-11-positive samples yielded two DNA fragments of 403bp and 560bp, respectively, in length for E2, E5, and L2 genes. The PCR produced another DNA fragment of 556 bp, of BPV\_11 gene in 4 udder teats samples that were amplified utilizing specific gene primers.



**Figure-3. PCR products for BPV-10 genotypes pictured in an ethidium bromide-stained 1.5% agarose gel following electrophoreses in TBE buffer. M: 100–10,000 bp marker; lanes: 1–8 BPV-10 positive samples (Teat wart samples) with band sat 403 bp; lane NC: negative control.**

**Table-2, showing PCR results of animals infected with teat and bovine udder papillomatosis.**

No. of sample	Age of animal (years)	Location of the warts	PCR results
1	6	Teats	BPV-10
2	5	Small growth on udder and teat	BPV-10&BPV-11
3	6.5	Small fleshy growth on udder and teat	BPV-10&BPV-11
4	5	Teats	BPV-10
5	7	Teats	BPV-10
6	5.5	Small fleshy growth on udder and teat	BPV-10&BPV-11
7	6	Teats	BPV-10
8	5	Teats	BPV-10
9	6	Teats	BPV-10
10	8	Small fleshy growth on udder and teat	BPV-10&BPV-11
11	5	Teats	BPV-10
12	7	Teats	BPV-10
13	6	Teats	BPV-10
14	7	Teats	BPV-10



**Figure-4, PCR products for BPV-11 genotypes visualized in an ethidium bromide-stained 1.5% agarose gel following electrophoreses in TBE buffer. M: 100–10,000 bp marker; lanes: 1–4 BPV-11 positive samples (udder and teat wart samples) with bands at 560 bp.**

It is clear from the above that PCR experiments ultimately showed mixed infections of viral DNA of BPV-10 and BPV -11 in four samples, and ten teat samples showed BPV-10 only (Table 2).

### Discussion

Figures 1 and 2 show the distinct appearance of benign skin tumor and multiple stages of hyperkeratinization in addition to other tumor related microscopic and gross changes in cutaneous layers. Previous studies by our team for different types of BPV (type 1, 2 and 13) showed that tumor samples were cutaneous fibropapillomas, which demonstrated the main features of papillomatosis<sup>(14,15)</sup>. PCR products for BPV-10 genotypes Fig.3 were detected with band sat 403 bp in agreement with<sup>17</sup> while PCR products demonstrated BPV-11 genotypes with band sat 560 bp (Fig. 4) in agreement with<sup>(18)</sup>. To summarize, PCR results identified either BPV-10 alone or BPV-10&BPV-11 (Table 2), with ten samples showing exclusivity to BPV-10 whilst four demonstrated a mixed infection of both BPV-10 and BPV-11. In brief, clinically diagnosed cases of the teat and bovine udder papillomatosis were studied to recognize BPV-10 and BPV-11 genotypes, which were prevalent mostly in the Middle part of Iraq that is the primary site for large cattle-raising farms. The detection and identification of BPV-10 and BPV-11 genotypes in this geographic area are crucial in effective disease control. In conclusion, Bovine papillomatosis of teat and udder were confirmed in this study to be initiated by BPV-10 and BPV-11 genotypes in the Middle regions of Iraq, where cattle farms are spread, which will ease the control requirements of the disease in the area.

### Ethical Approval

Consent was obtained from the owner of farm animals from which samples were gathered. Approval was obtained from the scientific committee at The Biotechnology and environment research center, University of Fallujah, Al-Anbar, Iraq. The experimental in vitro part was accomplished at the department of Experimental Therapy, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq

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