

## Article

# Clinico-Histopathological & Immunohistochemistry study of ruminant's cutaneous papillomavirus in Al-Muthanna Veterinary Hospital/ Iraq

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**Simple Summary:** Papillomatosis is widely spread oncogenic viral disease in ruminants in Iraq and lead in significant economic losses. This study intended to describe the clinical and histopathological features of papillomavirus in bovine, ovine and caprine, furthermore, to detect the expression of papillomavirus and the P53 protein using immunohistochemistry (IHC). Ten animals with multiple cutaneous papilloma lesions were included in this study. The exophytic multiple, cauliflower- growths (warts) were found on different areas of the animal's bodies. Various degrees of koilocytosis, ortho and parakeratotic, hyperkeratosis, hypergranulosis in granular layer and acanthosis were seen in histopathological sections. While, the IHC revealed intense positivity for papillomavirus antigen and P53. In conclusion, this study described the papillomavirus lesions in ruminants in Al-Muthanna governorate/ Iraq. PV antigen and P53 expression were positive in all tumor samples and can be considered as the useful markers in the diagnosis of cutaneous papilloma.

**Abstract:** Background: Papillomaviruses (PVs) are double-stranded DNA viruses and are more common in skin of ruminants in Iraq. A P53 (tumor suppressor protein) reveals an essential role in cell cycle control. This study aimed to describe the clinical, histopathological and immunohistochemical aspects of naturally occurring cutaneous ruminant's papillomatosis. Methods: Samples were collected from totally, 10 animals (3 cattle, 3 goats and 4 sheep) with multiple papillomatosis lesions. Results: Clinically, exophytic multiple, cauliflower-like growths (warts) of varying sizes (0.5-11 cm) were found in different areas of the animal's bodies. Histopathological features were various degrees of koilocytosis, ortho and parakeratotic, hyperkeratosis, hypergranulosis in granular layer and acanthosis. Immunohistochemical (IHC) investigations revealed some nuclei in the granular and basal layers of the epidermis with intense positivity for papillomavirus antigen. All tumor samples were positive for p53 expression that appeared as a strong cytoplasmic and perinuclear staining mainly in the basal and parabasal layers. Conclusion: this study described the papillomavirus lesions in bovine, ovine and caprine, that located in different anatomical areas with minor variations in histopathological features. The tumor samples showed positive results for PV antigen and P53 expression that considered as the useful markers in the diagnosis of cutaneous papilloma.

**Keywords:** Bovine cutaneous papilloma, Iraq, Immunohistochemical (IHC), hyperkeratosis, p53 marker.

## 1. Introduction

Bovine viral papillomas, commonly known as warts, are caused by the Bovine Papillomavirus (BPV), and result in proliferation of the skin and the development of cauliflower-like lesions [1,2,3]. The virus is primarily self-limiting, but warts may be removed either for cosmetic reasons or if the wart is irritating to the animal (e.g., near the eyes). The Bovine papillomavirus (BPV) is recognized as the causal agent of benign and malignant tumours in cattle, such as cutaneous papillomas, benign fibroplasias and urinary bladder & oesophagus cancer. It is causing significant economic losses [4, 5]. The virus prefers the stratified squamous epithelia of the warm-blooded animals like bovine. Simultaneously, only the horses and other equids revealed cross-species infection that reported in the literature [6]. Meanwhile, globally the viral infection occurs in all species of the animal and are highly species specific [1,5,6]. Bovine papilloma oncogenic virus has a double-stranded circular DNA genome of approximately eight kilobases [7,8]. BPV are non-enveloped, icosahedral symmetrical DNA viruses 50-55 nm in diameter from the papovavirus family (papilloma, polioma, vacuolating viruses). To date, 12 different species-specific serotypes of the virus have been reported. BPV-1 and BPV-2 are fibro-papillomaviruses related to the genus Deltapapillomavirus that show an affinity for epithelial and dermis tissue [9,10,11,12]. BPV-3, BPV-4, BPV-6, BPV-9, and BPV-10 are epitheliotropic and belong to the genus Xipapillomavirus. BPV-5, BPV-7, and BPV-8, conversely, belong to the genus-papillomavirus and cause both epithelial papillomas and fibropapillomas in the skin [13]. Under natural conditions, papillomaviruses are specific to the host, but BPV-1 and BPV-2 also infect horses, causing fibroblastic tumors [6]. Cutaneous papillomatosis does not generally pose a clinical problem, but they can sometimes become malignant, when accompanied by certain genetic and environmental factors. The infection can result in significant economic losses in animal husbandry due to lower milk, meat yields and reduced hide quality. Disease can appear on cattle of any age; however, it is seen more commonly and severely in animals less than two years old [8,14,15]. The primary source and natural carrier of the virus are cattle. The contagion enters the body through scratches or other defects. The infection occurs through both direct and indirect contact. Other factors that play a significant role in the occurrence of the disease are contaminated materials, milking machines, tuberculosis injections, malnutrition, and hormonal imbalances, as well as semen, mutations and long-term exposure to sunlight if there is immunodeficiency [16,17,18].

Diagnosis of infection is based on clinical symptoms, histopathological findings and the use of an electron microscope [19]. Another essential aspect of virus identification is polymerase chain reaction (PCR). There are numerous published studies done with FAP59/FAP64 and MY09/MY11 consensus primers designed based on the regions of the genome that code the structural proteins L1, L2, E6 and E7 [20,21,22,23]. The consensus primers are designed based on the region of the genome that codes L1 (HPV) in the human papillomavirus and are commonly used to identify the papillomavirus in humans, cattle and other animals [24].

A P53 (tumor suppressor protein) arbitrates several mechanisms in the cell such as cell cycle arrest, apoptosis, and senescence in response to specific cellular stresses (like various types of DNA damage, hypoxia, oncogene deregulation, oxidative damage and integrity, and repair) [5,25,26,27,28,29,30]. The significances of stress lead to translocates P53 from the nucleus of the cell to cytoplasm where, it interrelates with and controls membranes of the bcl- 2 proteins family, including bcl-2 itself. This mechanism results in the release of different pro-apoptotic proteins, that in turn activate apoptosis. P53 reveals an essential role in cell cycle control, hence, the loss of its suppressive function has been reported for various types of human neoplasia like squamous cell carcinoma and ameloblastoma [31,32] and animal tumours, like BPV induced tumours [5,26,30].

Papillomavirus is widely spread oncogenic virus in cattle, sheep, and goat in Iraq that is associated with benign and malignant lesions, and result in significant economic losses. Review of literature concerning the ruminant's papillomavirus lesions and detection the virus antigen and P53 expression in the tissue of the affected animals in Al-Muthanna governorate/ Iraq, revealed no previous publications. Consequently, this study intended to describe the clinical and histopathological features

of papillomavirus in bovine, ovine and caprine, furthermore, to detect the papillomavirus in the cutaneous papilloma lesions and the expression of P53 protein using immunohistochemistry.

## 2. Materials and Methods

### 2.1. Animals and Sample Collection

This study was approved by the animal and research ethical committee/ Al-Muthanna university (No.10. BPV/Oct/ 2018). The samples were collected from naturally occurring cutaneous papilloma or fibropapillomas in ruminants in Al-Muthanna governorate. The study was included 3 cattle, 3 goats and 4 sheep that presented to Al-Muthanna Veterinary Hospital during September 2017 to April 2018. The clinical examination was done on the affected animals that reveal a growth compatible with the classical growth of PV. All animals included in this study were showed multiple, cutaneous tumours localized anatomically in different areas such as the head, eyes, neck, back, testes, udder and muzzle regions. The samples were obtained surgically (Figure.1) after cleaning the area with water and soap and decontamination with 70% ethanol. Segments of warts were removed by the parallel incision in the surface of the skin using a disposable sterile scalpel and kept in sterile containers. Later on, the tissue samples fixed in 10% neutral buffered formalin and routinely embedded in paraffin wax, sectioned at 5 $\mu$ m and stained with Haematoxylin-Eosin (H&E) for histopathological assessment. The diagnosis was assessed following the guidelines proposed by [33].



**Figure 1.** Papillomavirus lesion, surgical removal and sample collection for histopathological and immunohistochemical investigations.

### 2.2. Immunohistochemistry

#### 2.2. 1. Detection of PV antigen

Sections (5 $\mu$ m) from the samples were labelled immunohistochemically by the streptavidin-biotin-peroxidase complex (ABC) technique for detection of the papillomavirus. Serial sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with hydrogen peroxide 3% in methanol for 15 min. The sections were rinsed with phosphate buffered saline (PBS, pH 7.2) and subsequently placed into citrate buffer (pH 6.0) in a microwave oven (800 W) for 10 min for antigen retrieval. After washing with PBS, the sections were then incubated with rabbit anti-papillomavirus antibody (Dako Cytomation, Glostrup, Denmark) for 30 min. Later on, the sections were incubated with biotinylated secondary antibody (Histostain®-Plus kit, Cat. No. AA85-9043; Invitrogen Corp, Camarillo, CA, USA) and Streptavidin HRP (Histostain®-Plus kit), each overlaid onto the sections for 10 min at room temperature. Papillomavirus positive cells were visualized using AEC and counterstained with Gill's hematoxylin. Primary antibodies excluded from the negative control sections, which were incubated either with diluted normal serum from the species in which the primary antibody raised.

#### 2.2. 2. Detection of p53 expression

Sections from the papilloma and two normal skin samples were immunostained using a streptavidin-avidin method (LSAB Kit; Dako). Paraffin sections of 4µm thickness were deparaffinized in xylene and 100% ethanol. The endogenous peroxidase activity blocked by 0.3% H2O2 methanol solution for 20 minutes. Then, sections were subjected to antigen retrieval with sodium citrate (pH 6.00) by heating in a microwave twice for 5 minutes each cycle (at 700W) and allowed to cool for 10 min. Later on, all sections were rinsed twice for 5 minutes in phosphate buffer saline (PBS; pH 7.4, 0.1 M). The block serum (Dako) was applied for 15 minutes to block non-specific bindings protein. The mouse anti-p53 antibody (NCL-p53-505, Novocastra) diluted 1:50 in PBS, was used for 1hour at room temperature in the humified chamber. Further, the sections incubated for 20 minutes at room temperature with the appropriate biotinylated secondary antibody. Following a rinsing step in PBS, streptavidin-conjugated to horseradish peroxidase applied for 20 minutes at room temperature. The colour development obtained by treatment with diaminobenzidine (DAB) (Dako) for 5 minutes. The sections counterstained with Mayer's hematoxylin. In the corresponding negative control section, the primary antibody was either omitted or replaced with appropriate normal serum. The examination of the immunoreactivity was determined in a "blind" study by two observers. The intensity of labelling in each specimen scored from absent to very strong immune-signal. Positive controls consisted of paraffin-embedded sections of human mammary cancer for p53.

3. Results

3.1. Clinical signs and gross appearance

In cattle, warts occurred on almost all parts of the body. All cases revealed the most popular classical types of papillomas that happened in the skin of cattle and reported in the literature. The age of the affected cattle was between 5 years and 6 months. The disease occurred in both sexes (2 female and one male). The lesions most commonly were located on the head, especially around the eyes, and on the neck, shoulders, perianal and external genital areas in the female, and also in the testes of the male (Figure.2 A& B; Figure. 3 A& B). additionally, they varied in size from 1 cm upwards, and their dry, horny, cauliflower-like appearance was characteristic. The lesions in all animals did not regress spontaneously, and warts persisted for more than 5-6 months and for two years in one case (according to case history), with serious loss of the body condition. One cattle in this study revealed a large lesion on the genital area on the vagina, which was infected and oozing pus (Figure. 4). The lesions on the testes appeared as single and sessile. Genital warts on the vulva and penis made mating impracticable because the lesions were of large size, friable, and bleed easily. All lesions were undergone surgical removal especially for the large lesions (Table. 1).

Table1. shows the clinical presentation of PV in the affected animals

No.	Animal species	sex	age	Anatomical locations of lesions
1.	Bovine	Female	5 years	perianal external genital, chest, abdomen, eyes, face and udder
2.	Bovine	Female	4 years	Face, shoulder, udder
3.	Bovine	Male	6 months	Face, around eyes, chest, testes,
4.	goat	Female	2 years	Udder, lips, eyes
5.	goat	Female	5 years	Lower lips
6.	goat	Female	4 years	External genital
7.	sheep	Female	4 years	Sternum , shoulder and perianal
8.	sheep	Female	3 years	Chest, perianal
9.	sheep	Female	4 years	Chest, sternum
10.	sheep	Female	5 years	Sternum and perianal

The disease in sheep appeared on the face, eyes, ears, lips, shoulder, chest, sternum and thigh (Figure. 5 A, B). Moreover, one sheep revealed deeply extended lesion on the sternum that reached the cartilage. Additionally, one ewe showed unhealed lesion after tough removal of warts from the thigh by the owner. The lesion was invaded with screwworms and continuously bleed, then the ewe became emaciated.



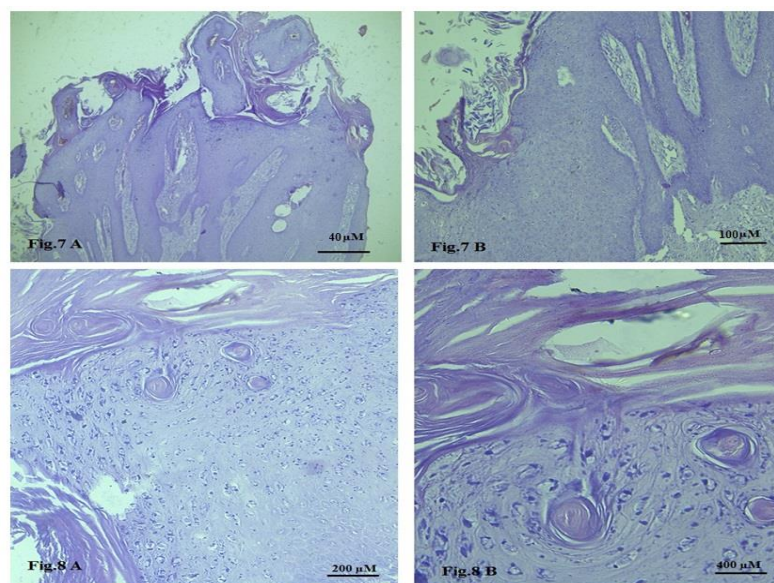
In goat, one animal revealed very large equal to the size of the apple lesion that located on the lower lips with deformity of the mandible and lost of the teeth. It was interference with prehension of food (Figure. 6 A). The lesions on the other goat were located in different body areas. However, the lesions in the perianal region were large and effected on the breeding of the animal (Figure. 6 B).



**Figure 2 A&B.** Shows the anatomical locations of papilloma in adult cow. 2 A: Multiple lesions on the abdominal wall of cow. 2 B: Multiple lesions on the eyes of cow. **Figure 3 A&B.** Shows the anatomical locations of papilloma in calf .3 A: A single lesion on the abdominal wall of calf. 3 B: A single and sessile on the testes of calf. **Figure 4.** Shows the large papilloma lesion located on the external genital of the cow. The lesion is infected and oozing pus and easily to bleed. **Figure 5 A& B.** Ovine papilloma lesions. A. The papilloma lesion located on the sternum. B. The lesions located on the eye. **Figure 6 A & B.** Caprine papillomatosis. A. A large (equal to the size of the apple) fibro-papilloma on the lower lip of the goat. B. The lesions in the perianal region were large and effected on the breeding of the animal

### 3.2. Histopathological features

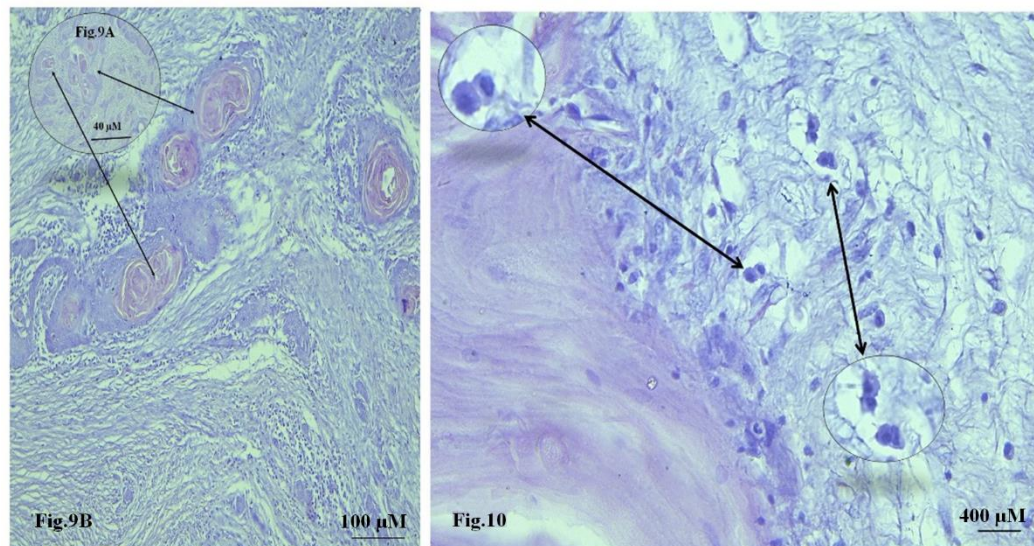
Microscopically, papillomas were characterized by well-developed finger-like projecting papillae with overlying stratum corneum, acanthosis, and uniformly down growing rete pegs (Figure.7 A, B). A varying degree of hyperplasia of the epidermis with irregular papillary projections into the dermis was common seen in all animals. Bovine cases were diagnosed as papilloma consisted of moderate to extensive degree of cornification (hyperkeratosis) with basket wave appearance, varying degree of parakeratosis, hyperplastic stratum spinosum with the presence of many koilocytes and islands of dermal connective tissue surrounded by hyperplastic epidermal cell layers (Figure.8 A & B). Basal cell layer was hyperplastic with hyperchromatic nuclei, moderate to severe mitotic activity and occasionally, invasive growth pattern was seen. Below epidermis, the neoplastic stromal tissue consisted of large stellate shaped fibroblast cells and intense fibrocellular proliferative changes (Figure.9 A&B, 10). The histopathological sections prepared from the large external genital papilloma (oozing pus and easily to bleed) revealed a large number of inflammatory cells invading the area (Figure.11A, B, C, D). The majorities were the macrophages and few polymorph nucleus. Comparable to cattle (with an abundance of koilocytes), sheep and goat diagnosed as fibropapilloma (exophytic) warts that showed only a few koilocytes 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 stroma and proliferated there extensively (fibropapilloma endophytic). Other diagnosed histopathological types were papilloma occult/ fibroblastic type and papilloma. Others showed variable degrees of ballooning degeneration (koilocytes) with the presence of abundant clumped, pleomorphic keratohyaline granules. Neutrophilic exocytosis into the dermis and epidermis were seen in the cases with secondary infection. In other cases, the epidermal proliferation was minimal and characterized as slight acanthosis and accentuation of rete pegs. Occasionally melanin granules were seen free within the subepidermal and within dermal melanophages. Numerous faintly basophilic to eosinophilic intranuclear inclusions measuring 10–15  $\mu\text{m}$  in diameter were present within keratinocytes of the exophytic, endophytic, and subungual cystic lesions (Figure. 12).



**Figure 7 A& B.** Shows well-developed finger-like projecting papillae with overlying stratum corneum, acanthosis, and uniformly down growing rete pegs. A. (X 4); B. (X10), H&E.

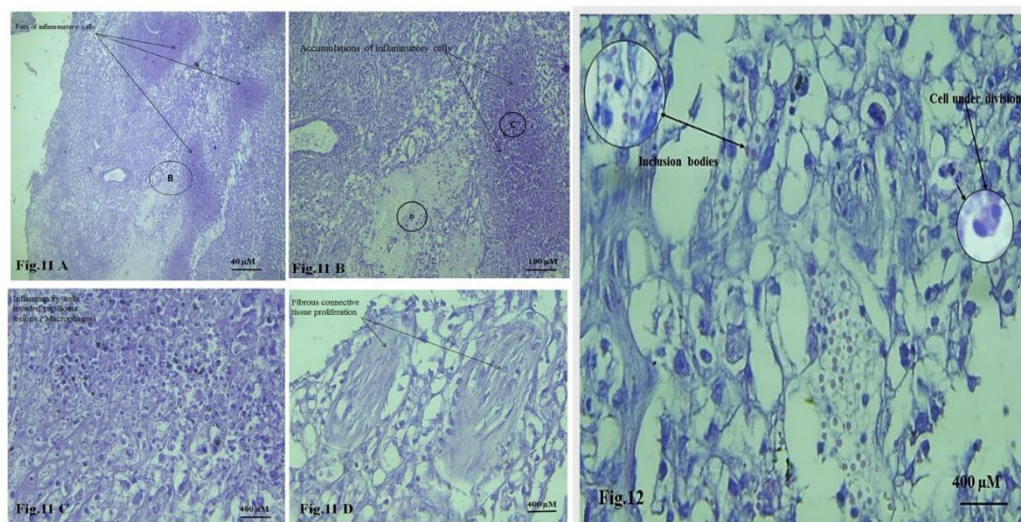
Figure. 8 A& B: Shows moderate to extensive degree of cornification (hyperkeratosis) with basket wave appearance, varying degree of parakeratosis, hyperplastic stratum spinosum with islands of dermal connective tissue surrounded by hyperplastic epidermal cell layers. A. (X 20); B. (X40), H&E.





**Figure 9 A&B.** Shows invasive growth pattern, and large stellate shaped fibroblast cells and intense fibrocellular proliferative changes. A. (X4); B. (X10).

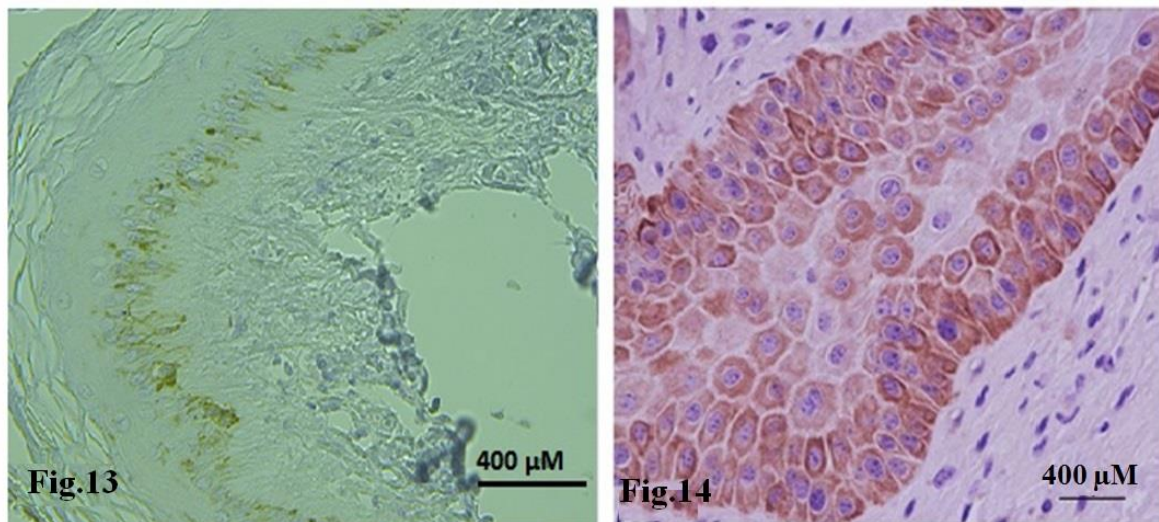
**Figure 10.** Shows the moderate to severe mitotic activities with divided hyperchromatic nucleus in the basal cell layer.



**Figure 11.** Shows accumulation of large amount of inflammatory cell the majority are macrophages. A. foci of inflammatory cells (X4); B. (X10); C. (X20); D. infiltration of inflammatory cells and fibrous connective tissue (X40). H&E.

**Figure 12.** Shows numerous faintly basophilic to eosinophilic intranuclear inclusions measuring 10–15 μm in diameter were present within keratinocytes of the exophytic, endophytic, and subungual cystic lesions. (X40, H&E)

### 3.2. Immunohistochemistry



**Figure 13.** Shows some nuclei in the granular and basal layers of the epidermis revealed intense positivity for papilloma virus antigen In the stratum corneum most of the nuclear remnants exhibited strong positivity (X20, H&E). **Figure 14.** Shows a strong expression of p53 protein that can be observed in the stratum spinosum, basal layer and parabasal layer (X40, H&E)

#### 4. Discussion

Bovine papillomatosis stands as a common viral disease of the skin, appeared as benign tumors or warts, caused by bovine papillomavirus (BPV) [18]. Papillomavirus may affect all ages of cattle; however, affected cattle were usually younger than 2 years of age [34]. In this study, cutaneous papillomatosis was detected in bovine, ovine and caprine and the age of the affected animals ranged between 6 months and 5 years. The lesions were detected mainly on the head and neck, and on the thorax. In some animals, the lesions were located within the other parts of the body and these clinical presentations are compatible with previous observations reported by other researcher [35,36,37,38]. Papillomatosis may become a significant herd problem when a large group of young, susceptible cattle becomes infected. The percentage of the disease may be increased approximately up to 20-25% [19]. In this study, papillomatosis was detected between two female and one young male in bovine with multiple distributions of the lesions on the body. The macroscopic and microscopic findings of the tumor that observed in the present study were similar to findings described before [15,37]. Macroscopically cauliflower shaped tumoral masses were observed. Histopathologic examination of the lesions revealed marked hyperkeratosis of the epidermis with irregular papillary projections into the dermis. Viral inclusion bodies are rarely reported in naturally occurred skin papillomas [35,39]. However, numerous faintly basophilic to eosinophilic intranuclear inclusions were seen within keratinocytes and tumor cells in the results of this study. This result is compatible with previous study [40], that demonstrated the presence of intranuclear viral inclusion body and the specific antigen (BPV-1) in the basal cell layer of the epidermis. Etiology of this disease is connecting to papillomaviruses, and cattle are the natural carries of the virus [14,34,39]. The observed histological features seen in this field cases were also in



agreement with the results of another study on bovine papillomavirus in the middle of Iraq [41].

Different markers can be used for determination the tumors proliferations. Moreover, the level of cellular proliferation in tumor tissue may be determined by immunohistochemical techniques. With these methods, a nuclear antigen associated with cell growth and division, stained and evaluated under the microscope [42]. The results of the immunohistochemical staining of the current study revealed intense positivity for papillomavirus antigen in some nuclei in the granular and basal layers of the epidermis. Moreover, strong positivity observed in most of the nuclear remnants of stratum corneum. These results are in agreement with previous studies that approved the extreme reaction for papillomavirus antigen [40, 43,44].

Because p53 is a useful marker of cell proliferation; they used several times in neoplastic skin tissues previously [40]. In the current study, the results of the immunohistochemistry showed that all papilloma and fibropapilloma samples expressed a strong perinuclear and cytoplasmic P53 proteins in the stratum corneum, parabasal and basal layers. This result reflects the accumulation of P53 in the cell cytoplasm that might occurred due to some considerable pathways and lead to disrupt its function as tumour suppressor. The strong expression of P53 has been documented in various human and animal tumours [31,32,43,46]. This result is also compatible with previous study that detected P53 cytoplasmic overexpression and perinuclear expression in equine sarcoids induced by BVP [5,6,26,47,48]. The strong overexpression of P53 in epithelial constituents of fibropapilloma has been proposed to occurred due to various stress factors consequent by a temporary increase of the normal P53 protein or accumulation of mutated P53 that is resistant to degradation or is not function [ 49]. Therefore, the result of P53 expression obtained in this study probably occurred because of impaired of its mechanism that result from neoplastic transformation due to BP oncogenic Virus. BPV is considered as causative agents of these tumors, inducing the proliferation of both epithelial and dermal cells [5]. Apoptosis is the tightly regulated process that plays an essential role in development and homeostasis, responsible for the balance between cell proliferation and cell death. The deregulation of the normal apoptotic process is considered as a “hallmark” of cancer [50,51,52,53,54]. Although few studies have stressed on a role for BPV’s oncogenes in harming some cellular pathways and leading to neoplastic transformation, nonentity is recognized about a possible interplay among the viral oncoproteins and P53 dysregulation. Therefore, the unraveling of new pathways possibly involved in the pathogenesis of bovine cutaneous fibropapillomas is of great importance to complete the molecular scenario triggering to the development of these tumors [2,25,52, 53,54].

## 5. Conclusions

The results of this study described the clinical and histopathological features associated with papillomavirus infection in bovine, ovine and caprine in Al-Muthanna governorate/ Iraq. Detection of papillomavirus antigen and the expression of p53 protein in the skin of the affected animals were

also approved. The authors recommend a further future study for better understand P53 function in vivo carcinogenesis of papillomavirus. Moreover, the correlation between farm animal papillomavirus and small animals should be determined. Specific treatment regime should be planned as the numbers of papillomatosis in the different animal species are increasing in Iraqi governorates.

#### Author Contributions:

All authors contribute equally in doing of this research and preparation of the article. Conceptualization, methodology, Writing-Original Draft Preparation, Karima A. Al-Salihi; Review & Editing, Ahmed H. Al-Dabhawi; Methodology, Ibrahim A. Erzuki; Methodology, Tho Alfiqar H. Ali.

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