

Pathological and Molecular Study of Bovine Papillomavirus 'BPV' in Basrah Province / Iraq

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ABSTRACT

The current study was conducted to detect bovine papillomavirus (BPV) in Basrah province in Iraq and study it pathologically and molecularly. Samples were collected (58) from veterinary hospitals in Basrah during the period from November 2020 to March 2021. The pathological distribution showed the prevalence of the disease among females by 41 (70.69%), while in males by 17 (29.31%), and the age of affected animals ranged between 1-3.5 years, the results showed that the age group ranged between 1-2 years the infection rate showed a higher incidence rate of 42 (72.42%) compared to the age group more than two years which showed 16 (27.58%), also the prevalence of BPV with irregular shape 51 (87.93%), while the circular shape was 7 (12.07%), location of the lesions on the animal's body, it was distributed as follows: neck 13 (22.41%), head 10 (17.24%), back 8 (13.79%), chest 8 (13.79%), mouth 7 (12.06%), thigh 6 (10.34%), Knee 3 (5.17%), Udder 2 (3.44%), eye 1 (1.72%). The results of macroscopical examination showed that almost all the skin lesions were external, which appeared in the form of a small, medium, or even large single wart and a lobular dome. These lesions were predominantly white to gray or even black in color, the lesions were of a rough and scaly nature. The microscopy results of BPV showed hyperkeratosis of the stratum corneum of the epidermis; also, there is a marked koilocytosis in the stratum granulosum and stratum spinosum as well to acanthosis accompanied with finger-like projecting papillae and rete pegs grown homogeneously. The molecular diagnosis was performed by DNA extracted of BPV samples, and then PCR was done, as the results of the molecular examination showed the presence of BPV conclusively in Basra, which were 52 positive cases and 6 negative cases were shown. Three isolates of BPV were presented for DNA sequencing. These isolates were successfully identified, and the specific sequence of these isolates showed the genotype of the virus, which was BPV type-1 in Basrah.

INTRODUCTION :

Papillomaviruses are circular, tiny, non-enveloped viruses belonging to the *Papillomaviridae* family, a diameter of 55 to 60 nm, and a double-stranded DN

genome of around 8 kilobase pairs(1), and have been linked to the occurrence of both benign and malignant lesions(2). Despite the fact that BPVs are species-specific viruses, BPV-1, BPV-2, and BPV-13 may infect equids as well as cattle and induce tumors in these animals (3).The virus has been found in numerous domestic species, including bovine, ovine, swine, felines, and canines(4). These viruses reproduce in the nucleus of squamous epithelial cells, and they have a strong affinity for skin and mucosal tissues, resulting in benign and malignant malignancies(5). There are 26 different forms of BPV, 23 of which have been categorized into five genera, and three forms that have yet to be characterized(6). BPV-1, BPV-2, BPV-13, and BPV-14 are the four forms of *Deltapapillomavirus*. *Xipapillomavirus* 1 (BPV-3, BPV-4, BPV-6, BVP-9, BPV-10, BPV-11, and BPV-15) and *Xipapillomavirus* 2 (BPV-3, BPV-4, BPV-6, BVP-9, BPV-10, BPV-11, and BPV-15) are the two species of *Xipapillomavirus* (BPV-12). *Epsilonpapillomavirus* 1 (BPV-5 and BPV-8) and *Dyoxypapillomavirus* 1 (BPV-7) are the other two genera(7). Finally, two newly identified kinds (BPV-17 and BPV-20) remain unidentified as species. BPVs are usually species-specific, however BPV-1, BPV-2, and BPV-13 may infect both cattle and horses(8). Cattle are the natural carriers and major source of BPV. The virus enters the body through scratches or other injuries, and illness spreads by contact with infected animals, both directly and indirectly. Contact with infected materials, milking equipment, and sperm appears to be another way for the virus to spread. Other conditions that cause immunodeficiency, such as malnutrition, hormone imbalances, mutations, and long-term exposure to sunlight, might increase the chance of infection(9). The ensuing viral illness, bovine papillomatosis, is characterized by cutaneous warts or papillomas, which are proliferative lesions varying in size from microscopic nodular lesions to giant cauliflower warts, which are generally rough and spiky to the touch and gray to black in color(10). Bovine papillomatosis has been observed in European countries like Italy(11), African countries like Nigeria(12), and American countries like Brazil(13) and Mexico(14), as well as several Middle Eastern countries like Turkey and Saudi Arabia(15). It's been reported in Iran as well(16). The existence of BPV-1 as the principal causal agent of bovine papillomatosis in Iraqi cattle was initially reported by (9). Although all ages can be infected with BPV, it is more common in calves and yearlings(10). Steers are less likely than heifers to be impacted (5).BPV diagnosis usually includes a clinical examination, histopathology, and immunohistochemistry(17). For the detection and genotyping of BPV, the polymerase chain reaction (PCR) has been utilized as a sensitive approach (18). BPV genotypes produce papillomatosis, a disease that causes severe economic losses in affected animals due to growth restriction, weight loss, and decreased milk output(19). According to(20), cattle are an important source of dairy and meat products in Iraq, and bovine papillomatosis causes major economic losses for Iraqi cow farmers (9).The importance of the illness and its expansion in Iraq necessitates research on the pathological, clinical, and genotypes that cause the illness in Iraq, which can aid in disease control.

MATERIALS AND METHODS

The samples were collected (58) from the main veterinary hospitals in Basrah province (Basrah veterinary hospital, Al-Medaina veterinary clinic, Al-Qurna veterinary clinic, Al-Zubair veterinary clinic) during the period from November 2020 to March 2021. The samples were transferred immediately to the laboratory of the pathology branch of the Faculty of Veterinary medicine at Al-Basra University . The

DNA was extracted from the samples using DNA extraction kit (Viral Nucleic Acid Extraction Kit II \Geneaid / Taiwan) . The gene was then amplified by PCR technique according to a special primers .(Table 1)

(Table 1) Primers sequence (9)

Primers	Sequence	Molecular weight
F	(5'-AGGAGGGTCATGCTTTGCTC-3')	847bp
R	(5'-GCTGTTCGGAGTGGTGTGTA-3')	

The PCR technique was applied by adding 5 µl from DNA extracted to the PCR tube containing 5 µl of the master mix and 1 µl of the F and R primers was then added to this PCR tube. In the end, 13 µl of the NFW were added to the tube to get 25 µl as a final size. The mixture then transfer to PCR system , the conditions of PCR reaction are listed in (Table 2).

(Table 2): The PCR amplification Condition

NO.	Steps	temperature	Time	Cycles
I	Initial Denaturation	94 °C	5 mins.	1
II	Denaturation	94 °C	45 sec.	35
III	Annealing	58 °C	45 sec.	
IV	Extension	72 °C	45 sec.	
V	Final Extension	72 °C	10 mins	1

Histological preparation of BPV was according to (21) .

Result

Distribution Results

The distribution results of BPV lesions of the current study showed that the characteristic pathological morphology of the papillomatous lesions appeared as irregular form in 51 (87.93%), while the circular form appeared in 7 (12.07%).The both forms mostly located in the neck of the animals that showed 13 (22.41%), then in the head 10 (17.24%); while other body site showed that the back 8 (13.79%) , chest 8 (13.79%), mouth 7 (12.06%), thigh 6 (10.34%), knee 3 (5.17%), udder 2 (3.44%) and the eye 1 (1.72%) as in table (3)

The distribution of the BPV lesions based on sex showed that the lesions appeared in female 41 (70.69%), while the lesions appeared in male 17 (29.31%).The results of age related lesions showed that the animal group that aged between 1 – 2 years showed higher incidence rate 42 (72.42%) compared to the age group more than 2 years that showed 16 (27.58%) as in table (3).

In addition, all the lesions appeared in both grey and black color spots of the animals, in which the grey regions showed 47 (81.03%) papillomatous lesions that appeared higher than the black spotted regions which showed 11 (18.97%). Besides, the results that the history of the routine treatment of BPV showed in small population of the studied animals which showed that the treated animal only 7 (12.07%) while that they did not treated 51 (87.93%) as in table (3).

Table(3) Distribution Results

Forms	Irregular	51 (87.93%)
	Circular	7 (12.07%)
Location	Neck	13 (22.41%)
	Head	10 (17.24%)
	Back	8 (13.79%)
	Chest	8 (13.79%)
	Mouth	7 (12.06%)
	Thigh	6 (10.34%)
	Knee	3 (5.17%)
	Udder	2 (3.44%)
	Eye	1 (1.72%)
	Sex	Male
Female		41 (70.69%)
Age	1-2 years	42 (72.42%)
	2-3.5 years	16 (27.58%)
Color	Grey	47 (81.03%)
	Black	11 (18.97%)
Treatment	Treated animals	7 (12.07%)
	Untreated animals	51 (87.93%)

Macroscopical Results

The lesions in the head, the neck, the knee, the udder and the thigh appeared as a solitary, rough and scaly with grayish colored masses ranged sometimes have a necrotized centers, the lesions ranged between 2 cm to 3 cm in diameter on the knee of the animal; that may coalesced to form a large solitary greyish mass that ranged 4 to 5 cm in diameter with rough scaly surface as in figure (1).

Macroscopical results of BPV lesions showed that almost of the cutaneous lesions were exophytic (proliferating outwards) that may occur in irregular to circular forms which appeared in various sizes were mostly located on the back of the animals which revealed as a solitary small to moderate dome shaped wart like mass, with a white to grayish colored lesions with a diffused multi-lobulated projections may coalesced together to form a big uniformed lobulated mass as in figure (1).

The lesions on the chest appeared somewhat large uniformed to coalesced grayish masses that have necrotized centers; the masses protruded downward like an irregular scaly to roughed cauliflower growth with marked hemorrhagic appearance as in figure (2).

The lesions on the mouth appeared as a large crowded to coalesced cauliflower like mass that contain a multiform white to grayish growth with a circular to irregular scaly and roughed uniformed mass as in figure (3).



Figure (1): Small to moderated dome shaped wart-like growth on the back region; it appeared as a solitary, white to grayish protruding masses (yellow arrows); that coalesced together to form a large uniformed grayish protruded mass (red arrow).

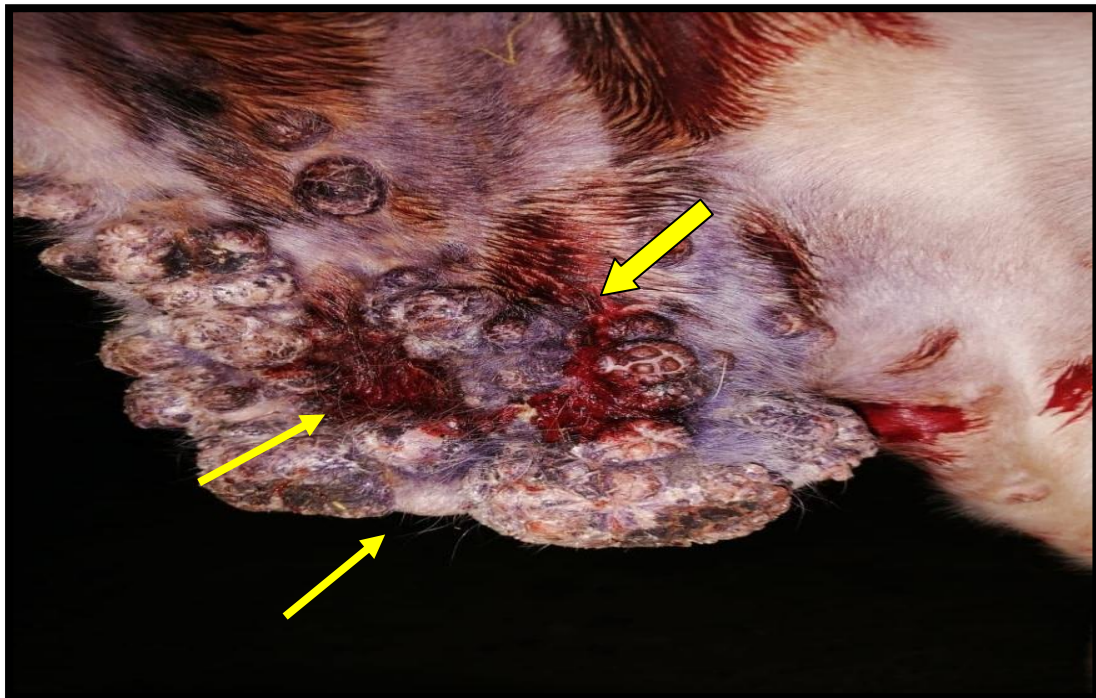


Figure (2): Large uniformed to coalesced grayish masses that have necrotized centers; that protruded downward like an irregular scaly to roughed cauliflower growth, with marked hemorrhagic appearance in the chest of the animal (yellow arrows).



Figure (3): Large crowded to coalesced cauliflower like mass that contain a multiform white to grayish growth with a circular to irregular scaly and roughed uniformed mass in the mouth of the animal (yellow arrows).

Microscopic observation

The microscopical results of BPV showed hyperkeratosis in the stratum corneum of the epidermis; also, there is a marked koilocytosis in the stratum granulosum and stratum spinosum as well to acanthosis. Also it shows a hyperkeratosis and acanthosis of the stratum corneum of the epidermis accompanied with finger-like projecting papillae and rete pegs grown homogeneously as in figure (4).

In addition, the other results showed single and small groups of cells with vacuolated cytoplasm referred to koilocytosis in the stratum spinosum of epidermis. The skin of BPV infected animal shows intra-nuclear inclusions bodies frequently located as slightly basophilic to eosinophilic structures in the keratinocytes of stratum spinosum as in figures (5).

The histochemical study in the skin of BPV infected animal shows large finger like projection of in the stratum corneum layer of epidermis revealed to collagen deposition in the center of the lesion surrounded by a thick capsular structure of keratin layer as in figure (6).

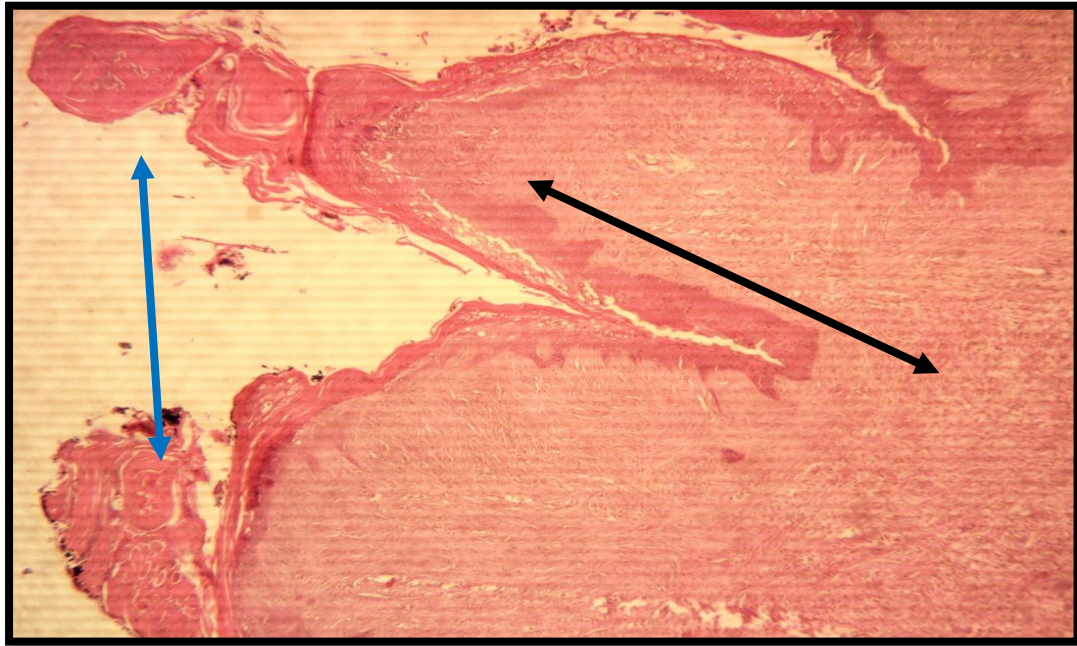


Figure (4): Histopathological section in the skin of BPV infected animal shows hyperkeratosis and acanthosis of the stratum corneum of the epidermis (blue dual head arrow); accompanied with finger-like projecting papillae and rete pegs grown homogeneously in the stratum spinosum (black dual head arrow). H&E stain. 10X

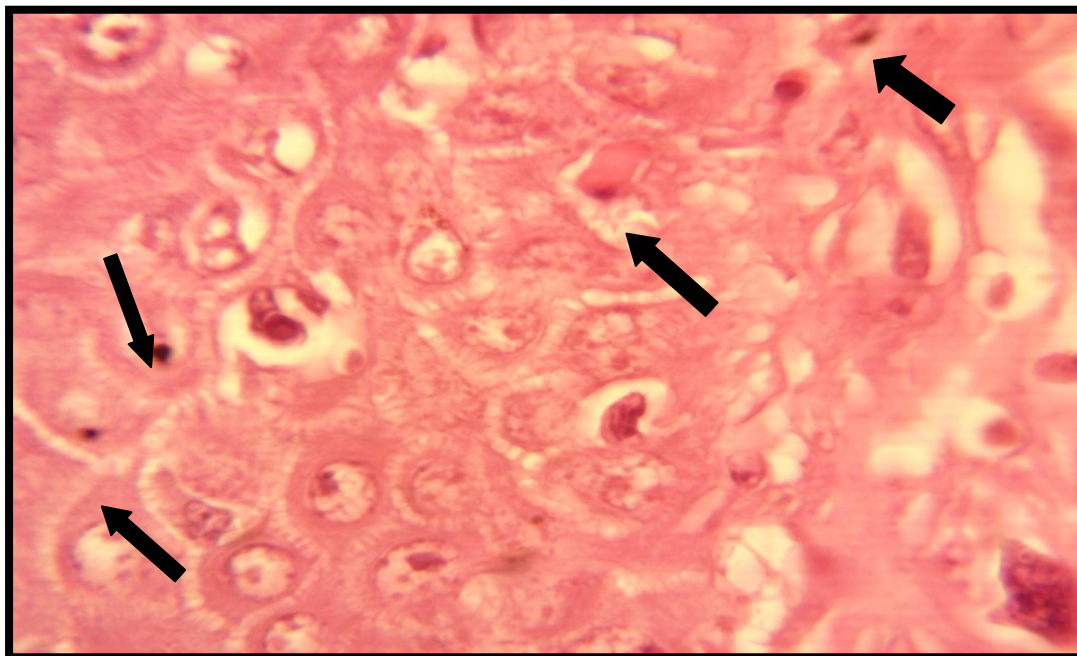


Figure (5): Histopathological section in the skin of BPV infected animal shows basophilic intranuclear inclusions bodies in the keratinocytes of stratum spinosum layer of epidermis (black arrows).H&E stain. 40X

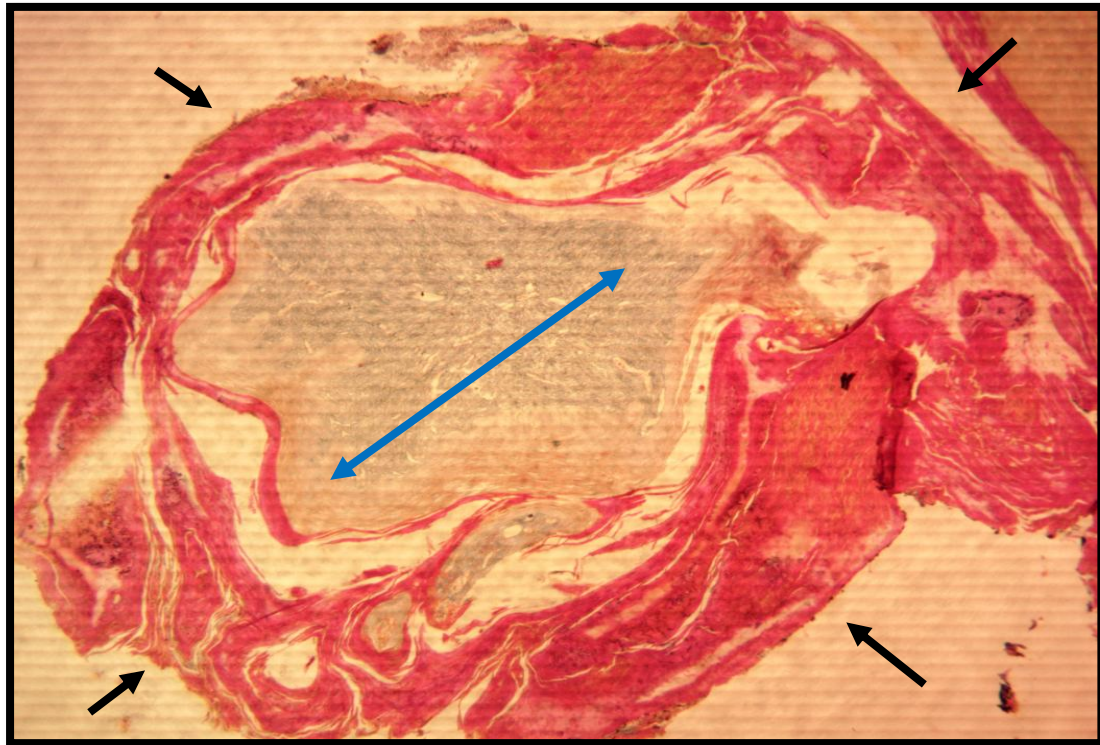


Figure (6): Histochemical section in the skin of BPV infected animal shows large finger like projection of in the stratum corneum layer of epidermis revealed to collagen deposition in the center of the lesion (blue dual head arrow) surrounded by a thick capsular structure of keratin layer (black arrow). Mallory trichrome stain. 10X

4.4 Molecular Diagnosis of BPV

The molecular diagnosis of BPV was done by the designed primer was amplified bands corresponding to its molecular size of approximately 847bp as in figure(7)

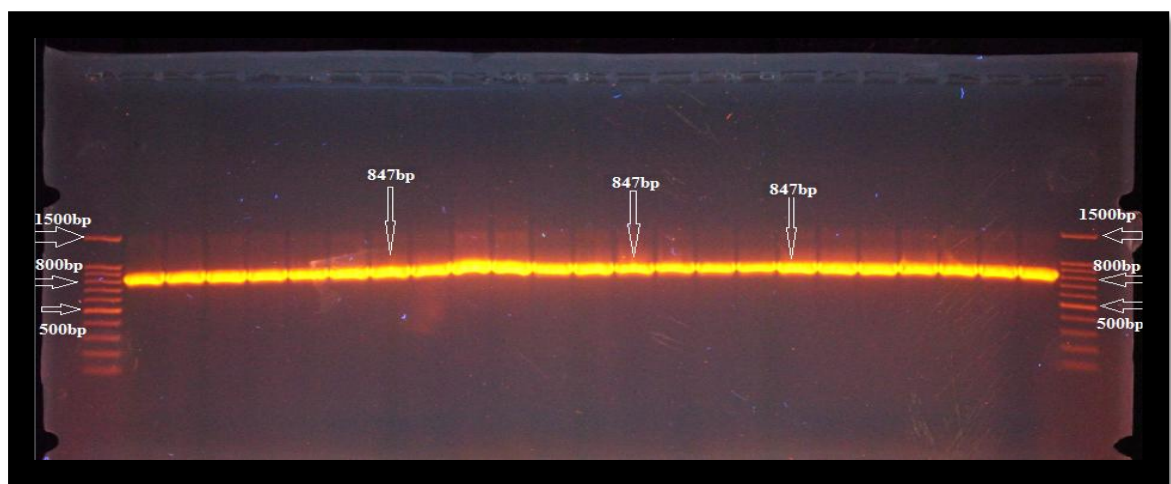


Figure (7): Agarose gel electrophoresis of PCR products Ladder = DNA ladder All Samples = positive samples (847bp).

4.4 Sequencing of PCR product

The sequence of the nucleotide of papillomavirus gene was known in 3 samples, as 25 microliters of each sample of the PCR product with the Primers of the nucleotide of papillomavirus gene were sent to Macrogen in the Korea and after obtaining the results (Appendix 2) all the results were compared directly with the nucleotide of the nucleotide of papillomavirus Available in the internet ([http: NCBI Reference Sequence](http://NCBI Reference Sequence)) by computer program (BioEdit Pro. version: 7.0.0). The results were registered in NCBI under accession numbers (LC606638) . Which is available on the website (<https://www.ncbi.nlm.nih.gov/search/all/?term=LC606638>).

(2)The most common types of mutations in the papillomavirusgene, sequence in this study.

No. of sample F	Wild type	Mutant type	Site
1	A	T	175
1	C	T	220
1	A	G	324
1	T	A	334
1	A	G	336
1	C	G	339
1	A	T	382
1	G	A	385
1	G	A	386
1	A	G	400
1	G	T	439
1	T	G	455
1	T	A	482
1	C	T	500
1	C	T	532
1	C	T	723
1	G	A	765
1	G	----	790

Discussion :

Distribution Of BPV:The current results showed that the main lesions appeared in the neck and head then in the chest, mouth, back, thigh, knee, udder and around eyes, because that BPV, the specificity of its tissues varies according to the genotype of the virus and the associated disease, in addition to the predisposing factors that make the disease more likely to arise such as, exposure to stress factors, exposure to sunlight, transmission between infected and uninfected animals , also contact between livestock, especially in housed feeding cattle than outdoor feeding .These results agreed with(22) they showed that the cutaneous lesions were proliferating outwards and these lesions of various sizes were mostly located on the head and neck, chest , back, thigh, knee, and sometimes spread other parts of the body in cattle , which In cattle, wart lesions are most commonly observed on the udder of lactation cows, as well as around the skin of the eyes, mouth, ears, neck, and occasionally on the skin of male and female genitals.This idea also agree with(23) who noted the neck was the most common site for lesions, followed by the back and right side of the face, and around the eyes, with the rest of the body being the least common. This is owing to the virus's site specificity, as well as animal husbandry and management practices.The current study indicate that most infection were found in young animals is higher compared to older animals, due to ill-developed immune system and in addition the young animals are more susceptible to parasite infestation and stress factors.which was agreed with(24) reported that the disease affect animals of all ages, but the highest infection rate occurs in younger due to acquired immunity from inapparent and apparent infection in the early stages of life, and that breed and sex play a role in infection incidence.The current study on BVP lesion based on sex showed that in female was higher than in male because the role of sex in infection and its increase in females might be due to stress factors such as pregnancy, lactating, and age progression, compared to males, where the process of fattening and slaughtering males at earlier ages, which was agreed with (25) reported immune suppression promotes papillomavirus infection, the animal's health and immune status are important factors in the occurrence of infection, and immunodeficiency can cause a delay in the healing of the wart lesion(26) who reported male cattle, on the other hand, are mainly destined for fattening and meat production, and are often slaughtered at the age of two years or less.Our study showed that BPV lesions with grey color higher than black because the disease passes through several phases from small nodular lesions to large warts, where in the phase of maturity the lesions turn from gray to black, so it may be in the presence of bleeding, which was agreement with(27) the resulting viral illness is bovine papillomatosis, which is characterized by cutaneous warts or papillomas, which are proliferative lesions that range in size from macroscopic nodular lesions to giant cauliflower warts that are generally spiny and rough to the touch and gray-black in color.In current study, some cases of BPV were treated by using an autogenous vaccine. The use of an autogenous vaccine to treat warts resulted in a high rate of lesions healing, because autogenous vaccine that was prepared by isolation and destruction of virus in infected animals and used to provide

immunity to the same animal, which was agreed with the result mentioned by(28) who mentioned on the efficacy of autologous vaccination in the treatment of warts .

Macroscopic observation:In current study showed macroscopic lesions vary from small to moderated to large dome shaped wart-like growth , solitary, rough and scaly with grayish colored masses,and some lesions also appeared as large uniformed to coalesced grayish masses that have necrotized centers; that protruded downward like an irregular scaly to roughed cauliflower growth, with marked hemorrhagic on the back region, head, chest, mouth, neck, thigh, knee, and udder of the animal , because this indicates untreated medium and large lesions that cause the prevalence the disease, which indicates the presence of small lesions on different parts of the animal's body.The presence of the lesion in a scaly and rough form indicates that the lesion is in growing phase and period has passed since the appeared of the lesion and disease on the animal.The presence of a solitary lesion in some areas indicates that the disease has not spread to all the parts of skin and also indicates that the infected part may have been exposed to stress factors or injury. The presence of bleeding from the lesion indicates an develop phase of wart, and immunodeficiency may be the cause of a delayed in healing of lesions , which was agreed with(29) reported papillomas were visually distinguished in different shapes and sizes, most of them were numerous and prominent above the surface of the skin as a cauliflower-like growth containing several cracks connected to the skin by a neck and some of them were in a single shape with a small size and a smooth surface.Also agreed with(30) who noted to warts in cattle were bumpy or cauliflower like masses of varying sizes and numbers projected from the skin of the head and neck, as well as the rest of the body.The surface of a wart is usually grey, rough, scaly and dry. Multifocal ulceration appeared on the top surface of the masses on occasion. Also agreed with (31)who reported fibropapilloma is a kind of cutaneous wart that is slightly elevated, dome-shaped, and exophytic in development, with an almost smooth or undulating outer surface, also described in cattle cutaneous warts were distinct from ordinary warts in that they were dome shaped or cauliflower like in appearance, with a smooth or undulating outer surface; they were described as occult papilloma/fibroblastic papilloma.Partially disagreed with(32) who noted greyish-white multilobulated outgrowths with a firm consistency were the skin suspected of papilloma .Our study agreed with(24) reported in cattle, the head, neck, shoulders, neck folds, limbs, and udder are common sites for papilloma and fibropapilloma. Tumors come in a variety of shapes and sizes, ranging from 1-2 cm to large structures that are rough, dense, and cauliflower-like. They can appear in almost any body area and have a typical papilloma appearance of variable sizes. Tumors are exophytic; sometimes that have a big attachment base or are pediculate .

Microscopic observation:current study microscopic results on skin sections related to infected animals with BPV showed hyperkeratosis in the stratum corneum of the epidermis accompanied with finger-like projecting papillae and rete pegs ; also there is a marked koilocytosis in the stratum granulosum and stratum spinosum as well to

acanthosis because of the replication of the BPV inside the epidermal cells lead to accumulation of keratin layer in the stratum corneum as well as to causing vacuolation in the epidermal cells, this idea may agreed with(33) who noted hyperkeratosis, koilocytosis, acanthosis, and hypergranulosis were seen in cutaneous papilloma and fibropapilloma, and morphological changes in the epidermis were similar to those seen in BPV infection, also agreed with(34) which mention that papillomas were characterized by welldeveloped acanthosis, and uniformly downgrowing rete pegs with finger-like projecting papillae and overlaying stratum corneum.The microscopical results also showed intra-nuclear inclusions bodies frequently located as slightly basophilic to eosinophilic structures in the keratinocytes of stratum spinosum , because BPV is DNA viruses and replicated in nucleus , that which agreed with(35) who reported intranuclear inclusion bodies were variable findings , and Partially disagreed with (15) who noted only the epidermis' basal cells observed a rare presence of intranuclear inclusion bodies.

In the current study histochemical results in the skin of BPV infected animal shows large finger like projection of in the stratum corneum layer of epidermis revealed to collagen deposition in the center of the lesion , surrounded by a thick capsular structure of keratin layer , because of over production and keratinized epithelial protein in the epidermis , that which agreed with(34) which was noted proliferation of cutaneous fibrous connective tissues and well developed finger like projections with connective tissue cores were showed.

Molecular Diagnosis of BPV:In the current study molecular examination, BPV-1 DNA was identified in 52 of 58 samples isolated by using polymerase chain reaction reactions (PCR) showed all band corresponding to a 847bp (9) .Our study according to the results, BPV with only one genotype is responsible for the majority of BPV infections in cattle. The six PCR negative samples of presumptive BPV specimens could be attributable to infection with other BPV strains than those studied in this investigation. Other investigations, however, have revealed the presence of coinfections (11), some of which have identified 2,3,and 4 BPV genotypes reported (36). In our study, however we only detected BPV-1 . This results could indicate that the animals are resistant to other genotypes or that they are only infected with BPV-1 once in their lives.

Sequencing :In current study amplification and sequencing of gene segments were used to molecularly identify the virus. As indicated, this study indicated a greater prevalence (97%) of BPV-1 infection, which was consistent with previous national or worldwide investigations (22) (36) (9) (38) .The results of sequencing of PCR amplicons of three isolates of BPV were identified to species level, the alignments of the virus isolates were identified and compatible and identified BPV-1 which belongs to the Deltapapillomavirus genus. This study discovered that Basrah has a high prevalence of highly pathogenic BPV-1, which is linked to the development of cutaneous papillomatosis (39).Also was agreed with (9) who noted the most of the

NCBI BLAST-searched BPV-1 sequences had 97 percent identity, confirming the presence of BPV-1. Amazingly, distance analysis revealed that this sequence had a significant degree of similarity to E5 amino acid sequences recovered from equine sarcoids in Egypt at 99% and from Bovine papillomavirus type-1 in the world at 97% . It may be the reason for a match with equine sarcoid in Egypt, it may go back to wars in the past when they used horses and donkeys, and the similarity between bovine papillomavirus type-1 with what is present in Iraq , Conforms globally compatibility due to the unlimited cattle trade with countries of the world.

conclusion

The susceptible age of BPV was 1-1.5 years and the head and neck and thorax were the most sensitive area for BPV infection and mainly in the female .BPV led to series macroscopical and microscopical lesions ranged from solitary to multiple wart like growth consisted hyperkeratosis and other characteristic . The molecular detection of BPV identified the presence of BPV in Basrah as well as the sequencing identified the close similarity to Egypt strain .

Acknowledgements

I am greatly indebted to my parents, brothers and sisters for their support, understanding, prayers, love, patience and sacrifices rendered during my life and study to make this day possible...

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