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Clinical and Molecular Study of Bovine Ulcerative Mammillitis in Cattle in AL-Qadisiyah Province

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

فَتَعَٰلَى ٱللَّهُ ٱلْمَلِكُ ٱلْحَقُّ ۗ وَلَا تَعْجَلْ بِٱلْقُرْءَانِ مِن قَبْلِ أَن يُقْضَىٰٓ إِلَيْكَ وَحْيُهُۥ ۖ وَقُل رَّبِّ زِدْنِى عِلْمًا ﴿١١٤﴾

صَدَقَ ٱللَّهُ ٱلْعَظِيمُ

سورة طه

Abstract

Bovine ulcerative mammillitis (BUM) is endemic disease of cattle in many countries, including Iraq. It is causes by alpha-herpersvirus that induces an infection of skin of cattle, which develop in several possible pathological forms: server acute ulcerative form, superficial dermatitis with alopecia and crusts, and pseudolumpy skin disease. It is important to be studied because of its transmissible nature and has economic impact on the dairy industries. The current study focused on two parts, the first part was to determine epidemiological and clinical elements, and the second part concerned on the molecular diagnosis and phylogenetic study in AL-Qadisiyah province of Iraq, but the narrow period of study and delayed of sequence results prevent included some results.

In the first part of study, clinical examination and observations to determine the BUM in 200 adult lactating cattle (100 crossbred and 100 local breed) and 50 calves from Al-Qadisiyah province, clinically, the results showed that 18 (7.2%) animals out of 250 examined cattle were having teat, udder and muzzle lesions. Thirteen (6.5%) of dairy cows were infected, all infected cattle were at lactation period. Five (10%) calves aged from 1 week to 3 months were have muzzle and mouth lesions the lesions teat and udder in dairy cattle and muzzles of calves. Lesions superficial dermatitis caused by bovine herpesvirus 2 (BoHV-2) in dairy breed calves. Clinically, all of the affected cattle were 2-6 years of age, had ulcerations and crusts on the clinical signs that have been observed included sever from acute clinical lesion, characterized by enlargement and increased in size of one or more teats, usually the infection effected four teats and extended the lesion to skin of udder. in general the severity of lesions ranged from several ulcers covered with tightly adherent of yellow to orange color scabs to sever acute lesions involved whole the teats with irregular shape of ulcers, scabs and cracks of teats.

Polymerase chain reaction results detected BoHV-2 gene in all clinical samples (7.75%). All 18 of the seropositive samples were PCR positive, molecular detection was targeting *gb* enes which gave band corresponded to 607bp segment.

In this survey, depending on the molecular results, some epidemiological elements as age and breeds to BoHV-2infected were also investigated. To analyze the risk related with cattle breed associated with the BoHV-2 infection, two breeds (crossbred and local breed) of Iraqi cattle were infected. Both breeds were infected at percentage 8 (8%) and 5(5%) for local breed and crossbred respectively. Statically the results revealed that there were no significant differences them for their susceptibility to BoHV-2infection. The results of this study showed that the number of infected animals decrease with age The infection rate among the cattle of 2-4 years of age group was 9/110 (2.8%), whereas of cattle over than 5 years old 4/90(4.4%). Also results revealed that there were significant differences between ages of cattle for their susceptibility to BoNV-2.

In conclusion, these isolated virus in these Iraqi isolates are members of the BoHV-2 family and indicated by clincal, molecular, These Iraqi BoHV-2 isolates represents an invaluable tool as a source for future studies and provide a reproducible cell culture system for studying the herpesviruses.

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1-1- Introduction:

Bovine ulcerative mammillitis (BUM) is a chronic transmissible viral disease of cattle (Lairmore, 2014). It is characterize by a skin and skin associated strictures disorder (EFSA, 2015). The disease is associated with infection by Bovine ulcerative mammalitisvirus (BoHV-2), which also recognized as a long-latent infection virus in cattle (Kettmann, *et al.*, 1994 and Miura, *et al.* 2015). Bovine ulcerative virus is characterized by a very long latent period, most infected cattle appeared asymptomatic carriers (Tsutsui, *et al.*, 2016). Most of BoHV-2 infected cattle develop persistent infection.

The disease is transmitted by horizontal way, horizontal occurs by direct contact, and inappropriate management practices (Kobayashi, *et al.*, 2010, Ooshiro *et al.* 2013, Lairmore, 2014 and Kobayashi, *et al.*, 2015). Bovine ulcerative mammillitis virus is an economically important viral infection of cattle (Erskine, 2012). The estimated costs of BoHV-2 infection in United States were 525 million dollars annually, due to lost productivity and premature death, while the average reduction in annual value of production was $ 59 per cow for BOHV-2-positive herds (Ott *et al.* 2003). In mid-Atlantic dairy herds, (Rhodes, *et al.*, 2003) referred to that 412 dollars the cost of case of LP, while mean annual cost of subclinical infection at a 50% prevalence of infection was 6,406 dollars per 100 milking cows.

Other causes were reported that associated with the decreased milk production, the National Animal Health Monitoring System (NAHMS) study determined that herds with BoHV-2 produced 3% less milk, than non-BoHV-2 herds (Ott *et al.* 2003). Decreased reproductive efficiencies, But a major indirect cost associated with infection is the restriction of the sale of animals (Pelzer, *et al.*, 1997). The cost of control and

eradication varied with herd size, the mean annual cost of a test-and-manage control program was 1,765 dollars with a 50% prevalence of BoHV-2 infection (Rhodes *et al.* 2003). In Canada, a total annual treatment costs of EBL infected 50 cows was $ 806 dollars (Chi, *et al.*, 2002). Subclinical mastitis, increased morbidity, decreased resistance to other diseases were also reported (Kale *et al.* 2007).

The disease was reported in Iraq neighboring countries, where BoHV-2 infection in dairy herds of northeast of Iran was reported (Mousavi, *et al.* 2014). The BoHV-2 virus infection was recorded in Turkey (Alkan, *et al*., 2011and Sevik, *et al.*, 2015). Moreover, Saudi Arabia has confirmed cases among local traditional and dairy cattle (Hafez, *et al.*, 1990). In Jordanian dairy farms, (Ababneh, *et al.*, 2012) documented the presence of two BOHV-2 genotypes (Schwartz, and Levy, 1994 and Kale, *et al.*, 2007).

In spite of the serological and molecular methods which were faster, easier than culture and the false positive and false negative are excepected results (Choi, *et al.*, 2002 and Mohammadabadi, *et al.*, 2011). The isolation of BoHV-2 still important and appropriate method to confirm the disease exist, and study of viral peculiarities, disease pathogenesis, and also provided a vital source to *in vitro* and *in vivo* experimental models (Chatterjee, *et al.*, 1985, Suzuki, *et al.*, 2009).The studies on BoHV-2 infection has not been conducted in the middle Euphrates in Iraq. Therefore the present study was conducted to provide the preliminary data about diagnosis of the BoHV-2 in these areas and obtain additional clinical and epidemiological pivots. This study links with the programs of the disease control, the viral isolation of Iraqi virus strain, source of virus, focusing on vaccination strategies for cattle protection and provides a vital source for experimental models.

1-2- Aims of the study:

Because of the worldwide distribution and high economic importance of the disease, and the long life infection with asymptomatic feature, that precipitate the challenges for this study was to dtection the causative agent of BUM from cattle located in the AL-Qadisiyah in Iraq by using the best diagnostic tools for detection of the BoHV-2 infected cattle. Therefore this study was aimed to: _

1. Obtaining additional clinical and epidemiological data about the BoHV-2 infection in Iraq.
2. Molecular and phylogenetic characterization of BoHV-2

2- Literature review:

2-1- History of Bovine ulcerative mammillitis (BUM):

Bovine ulcerative mammillitis is a contagious disease of cattle showing ulcerative, vesicular, and erythematous lesions mainly on teats and udder (Gillet, *et al.*, 2007). At the beginning of the 20th century, pathologists interested in this disease observed that BUM is a herd disease of probable infectious origin (Moratoia, *et al.*, 2010). Bovine herpesvirus-2 which belongs to the family *Herpesviridae* which causes bovine mammillitis and pseudo-lumpy skin disease. Bovine ulcerative mammillitis is often associated with cold weather and in first lactation heifers. (Chiba, *et al.*, 1995).

According to (Miller, *et al.*, 1969) the first isolation of BoHV-2 as a causative agent from lumpy skin disease infected cattle in southern Africa (1) subsequently the virus has been isolated from cases of mammillitis in other European countries and Australia (3) observed that BOHV-2 particles are small circular single strand DNA exogenous, that allowed the classification of BoHV-2 amongst the gammaherpes viruses. The other successful attempts to find the virus was during cultured cell derived

from the skin cells and electron microscopic examination, the virus was designated as bovine ulcerative mammillitis virus and exhaustive epidemiological studies proved that is was the causative agent of bovine skin lesions (Ferrer, 1980 and Lairmire, 2014).

Reichart, (2002) referred to BUM as a chronic disease of cattle which develops over a long period of time, the disease cause by bovine herpes virus. Today, with the exception of some European countries, BoHV-2 became globally wide spread and a cosmopolitan cattle disease (Niecvmann and Buchring 1997 and Tsutsui, *et al.*, 2016). Also, Suzuki, *et al.*, (2008).

2-2-Bovine ulcerative mammillitis:

Bovine ulcerative mammallitis is one of the important diseases which listed by the World Organization for animal health (OIE) as a disease of importance to international trade (OIE, 2012). develops in a minority of infected animals over a long period of latency (Gillet, *et al.*, 2007, and Matsvoka and Jeand, 2011). Unlike SBL, etiologically, BUM is associated with BoHV-2; which is the most common neolatent state astic disease of cattle, the vast majority of infected animals remain healthy with no outward signs of infection and no apparent negative economic effects (Miyasaka, *et al.*, 2013, Ochirkhuu, *et al.*, 2015).

2-3-Etiology: -

Bovine herpesvirus -2 is the causative agent of BUM, also termed Bovine leukosis virus (Murphy, *et al.*, 1999). BoHV-2 belonged to a dultaretrovirena which one of six subfamily *Bovine alphaherpesvirus 2* species (BoHV-2; order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Simlatent state exvirus*) is the cause of ulcerative mammillitis, which is usually a self-limiting cutaneous disease of the udder and teats. Morphologically, BOHV-2 can consider as C- type enveloped virus derived from the host cell membrane, are spherical shaped with a diameter about 80–

100 mμ. Consist of Three layered structures, the innermost is the genome nucleoprotein comlatent state ex, which includes about 30 molecules of reverse transcriptase, which is enclosed within an icosahedral capsid protein, Some particles presented with intermediate membrane in close apposition to the nucleoid, the virus dislatent state ay unique peculiarities if primary affected lymphoid tissue of dairy and beef cattle, (Scwartz and levy, 1994, Ott, *et al.,* 2003 and Hamard-Peron and Muriaux, 2011).

Ferrer (1981) suggested that the relatent state ication of virus occurs in the cytolatent state asm through the formation of CDNA as intermediate step and the initial formation of small aggregated structures of virus and they observed free viral particles and budding structures of mature virus from cell surface membranes of infected cells.

Gillet, *et al.*, (2007) and Maertens, (2015) stated that Structural and biological properties of BoHV-2 seem to be related with BoHV-1, BoHV-3 and BoHV-5, which are notably similar with an absence of chronic viraemia and with long latency period deelatent state y also there are functional relative resemble with highly clear cut sequencing homologies (Schwartz and Levy, 1994, Rodriguez, *et al.,* 2011 and Yida, *et al.,* 2013).

2-4- Epidemiology:

2-4-1 Transmission:

Bovine ulcerative mammillitis virus is a transmissible disease could be vertically and horizontally transmitted from infected animal to the susceptible animals, the transfer of BoHV-2 infected cells via several potential routs is essential for dissemination of the disease (Monti, *et al.,* 2007, Ooshiro, *et al.,* 2013). BoHV-2 transmission occurs via the transfers of BOHV-2 infected cells that are present in blood, milk and body secretion from BoHV-2-positive animals to the susceptible host (Gutierrez, *et al.,* 2014). A small volume of whole blood from an infected cow is possible to transmite BOHV-2 infection to healthy animals through various routs intramuscular, intravenous, subcutaneous, or intradermal (Lairmore, 2014). Also other

researcher suggested the possibility of horizontal transmission should be considered because of the infected lymphocytes are present in the biological fluids, and this risk of transmission is increases in confinement situation when present of fluids and tissues especially during calving (Lassauzet, et al., 1991 and Boxer, 2002). BoHV-2 transmission is more effective when the infected animals are in the latent state stage, because most cattle with latent state may have more 35% of infected lymphocytes harboring virus, compared with that cattle without latent state which have less than 5% infected lymphocytes (Tuliarena, *et al.,* 2007). Also (Pollari, and his group, 1992) referred that the virtue of distribution of BOHV-2 in herd had greater milk production more likely.

Because of the vast majority of cells becoming infected at early stage during the initial infection, the primary infection is crucial period for BOHV-2 transmission (Gillet, *et al.,* 2013).

2-4-1-1- Horizontal Transmission: -

There are various ways to achieve the horizontally transmission of BoHV-2, this way of transmission has been recognized as a major infection route for BoHV-2, and animals with high viral loads are considered to be a major infectious source in cattle herd (Mekata, *et al.,* 2015) (i) direct contact: long period closed contacts between animals represents one of the important way that occurs examlatent state e as broken skin and mucosal surfaces, through tissues, blood and exudates (Ooshiro, *et al.,* 2013). Also Contact transmission occurs via a mixture of natural sources, blood, and exudates as nasal secretion and saliva (Yuan, *et al.,* 2015).

Another possible method may latent state ay a major role in BOHV-2 transmission within herds are (ii) Hematophagous Insects (Manet, *et al.,* 1989). Also (Bech-Nielsen, *et al.,* 1978 and Burny, *et al.* 1986) status that the worldwide distribution of BOHV-2 in temperate climates and in countries with heavily populated

by haematophagous insects can occur, also it transmission is highly prevalent during summer months compared with winter months that gives strong association with these activity. (Kobayashi, *et al.,* 2015) referred to the risk of infection of the uninfected cattle that neighboring to infected cattle, had a significant higher risk of seroconversion than those without any infected neighbors.

(iv) Herd Management Practices, Dehorning is considered one of the most management practice in dairy herds, some Iranian studies recorded that dehorning with contaminated instruments has increased the risk of transmission of BOHV-2 (Haghparast, *et al.,* 2012). (Erskine, *et al.,* 2012) suggested that the management latent state ans and some risk factors that may can be important in transmission of BOHV-2 within herd (DiGiacomo, *et al.,* 1985).

2-4-2- Occurrence of BoHV-2:

Bovine ulcerative mammillitis" which was reported in different countries like Great Britain, Australia and United States. Hence, the term

The data of epidemiological studies has been suggested that BOHV-2 infection is globally distributed, and can be affecting bovine of all breeds and ages (Benavides Benavides, *et al.,* 2013). Bovine ulcerative mammillitis is pandemic disease, and it has high variable prevalence among countries. Although voluntary control programs are in latent state cattle in the USA, prevalence is high compared with much of the rest of the world, Also in the USA, most recent surveys estimate that 10% -14% of beef cattle and 38% - 44% of dairy are infected with the BOHV-2. Prevalence tends to increase on dairies with increasing herd size, while the converse is true in beef cattle (Lomonaco, *et al.,* 2013). Also in Canada BOHV-2 represent a major constraint disease to cattle trades, In Denmark, BOHV-2 studies showed that the spread and occurrence of disease followed the vaccination campaigns of infectious diseases (Gottschau, *et al.,* 1990)

In general, the true prevalence of BOHV-2 in South Africa is present at low prevalence, except in some regions it was determined to be 70% (Hestevbrg, 2007). In Argentina, BOHV-2 infection is considered an endemic disease especially in dairy herds of the northern and central areas, in highly infected herds about 80 % of cattle seropositive to BOHV-2 (Da, *et al.,*1993 and Ghezzi, *et al.,* 1997). And within herd the prevalence ranged from 40% to 50% (Trono, *et al.,* 2001), While the prevalence of disease in Colombia was reported as from 19.8 - 40 % (Alfonso, *et al.,* 1998 and Benavidies, *et al.,* 2013).

The epidemiological situation of BOHV-2 prevalence in Asia is more uncertain, the BoHV-2 is recorded in Indonesia, China and Mongolia (OIE, 2015). Seroprevalence rates in Japan were found to be 28.6% and 68.1% at the individual and herd levels respectively (Murakami, *et al.,* 2011). (Suh, *et al.,* 2005) demonstrated that the individual seroprevalence rates exceeded 50% whilst 86.8% of dairy herds were infected In Korea, (Choi, 1982) was the first detection of the EBL in Korea by the serological method was in the 1982, also the recent prevalence in dairy cattle was found to be over 50% (Chu, *et al.,* 2007 and Jung, *et al.,* 2012), in other countries Cambodia and Taiwan only about 5% of animals were positive for BOHV-2 (Wang, *et al.,* 1991 and Meas, *et al.,* 2000).

Regarding the Middle East countries, all reports referred to that the prevalence of BOHV-2 infection is somewhat lower than in other countries of the world (Pourjafar, *et al.,* 2004 and Trainin, *et al* 2005). Except in the regions of Iran and Turkey The degree of spread and prevalence of BOHV-2 in these neighboring countries of Iraq have some importance. In Turkey (Meas, *et al.,* 2003) referred to the restricted spread and low percentage of 1.6% in some cattle herds. In a recent study in Iran, on the level of herds infectivity a study of detected seropositive by ELISA which was 38 (41, 3%) out of 92 herds were infected (Haghparast, *et al.,* 2012). In another study the results showed

that 109 (25.4%) out of 429 serum samlatent state es were BOHV-2 seropositive and in other the individual seroprevalence in Iran was estimated between 17 and 24.6% (Hemmatzadeh, *et al.,* 2007), while herd prevalence was 64.7% (Gonzalez *et al.,* 2007) in another investigation in five Philippinean islets. The prevalence varied dependent on tests assay in the BOHV-2-CoCoMo-qPCR-2 was 108 (9.7 %) positive for BOHV-2 provirus while 54 (4.8 %), were determined by nested PCR (Polat, *et al.*, 2015).

Also in Iran, Mohammadi *et al.,* (2011) and Nekoei, et al., (2015) suggested that 29.9% and 22.1% respectively for BOHV-2 seropositivity was found among the samlatent state ed cattle. Previous report on Holstein cattle in slaughterhouse the seroprevalence rate was 22.3% (Tooloei *et al.,* 2009). The prevalence of BOHV-2 infection in a clinically healthy Holstein cows from a commercial dairy herd in southern Turkey was 59.6 % (**Kale,** *et al.,* 2007). And the prevalence of infection in dairy cattle in Israeli is low, the main national infectivity is about 5% and approximately 60% out of tested herd were BOHV-2 negative reactor (Brenner, *et al.,* 1989).

The clinical BUM is rare in New Zealand due to restriction of cattle and genetic material importation, (Thompson, *et al.*, 1993) referred to BOHV-2 infection of 5-10% at herd level Tan,, *et al.*, (2006) tested 313 serum samlatent state es collected from 5 dairy cattle herds, for the presence of antibodies against BOHV-2 using virus neutralization (VN) and agar gel immunodiffusion test, the results indicated that the prevalence of BOHV-2 infections was between 19.5% and 0.3%. While Scoepf, *et al.,* (1997) demonstrated that the 2047 of dairy cattle and 802 beef cattle were examination in Tanzania using the ELISA test to assess the BOHV-2 prevalence which approximately was 41% and 21.4% of dairy and beef cattle respectively with an overall prevalence was 36%.

The gamma herpes viruses is considered highly prevalent in Asia, the overall prevalence of BOHV-2 was 28.7%, in that the prevalence of dairy and beef cattle were

34.7 and 16.8% respectively (Murakami, *et al.*, 2011 and Giovanna *et al.*, 2013). In New Zealand and Australia have eradication programs in latent state ace that have led to negligible rates of BOHV-2 infection (EFSA, 2015).

2-4-4- Risk Factors:

The infected cattle are the only source for infection to BoHV-2-negative animals and, in general, a herd becomes infected when BoHV-2 positive cattle is unknowingly incorporated into the negative herd. Routine management practices on dairy farms cause BOHV-2-dissemination (Scott, *et al.*, 2006). Without serological or viral tests performed, the disease which stays unnoticed for several years. Such silent transportation allows BOHV-2 to reach a high prevalence before the pyramids top point dislatent state ay with clinical detection of the first lymphosarcoma/leukemia and then the comlatent state ete pyramid is then discovered by hematological and serological assays.

Management practices:

Herd management practices can be considered as important factors managed the transmission of disease, the prevalence of BOHV-2 in herds with the same number were 32.8% and 73.6%, the reason for deference in prevalence may be due to a loose of housing system can increase the physical contact among cattle and loose of insect control program (Haghparash and Mohammadi 2012).

Lucas, *et al.*, (1985) and Dimmock, *et al.*, (1991)who mentioned to several ways of iatrogenic technique may be contribute in the transmission of BOHV-2 such as vaccination, drugs injection, dehorning, tattooing and castration that carried out with minimum hygiene.

2-4-5- Economic Impacts:

Bovine ulcerative mammallitis causes a decline in milk and may be secondary mastitis and resultant culling of productive animals.

In Japan farmers claim Leading to serious economic production in dairy cattle considerate one of production limiting diseases in would (Erskine, *et al.*, 2012). Studies about the economic effects of EBL infection on production is variable, it involved the direct and indirect economic impacts (Trainin and Brenner, 2005). The first included the cost effective BOHV-2 control program, cost association with diseases effect, cost of prevention, cost of treatment of asymptomatic cases (Pelzer, 1997). The epidemiological conclusion of results of previous and on-going research about BOHV-2 consequences influences in reproductive and performance in dairy and beef cattle, the invisible losses of productivity have a significant value in economic impact on the animals industry, the disease has been a major economic significance as highly losses are due to drop in milk yielded in dairy herds (Chi, *et al.*, 2002).

Establishing the interrelationships among BOHV-2 infection and annual value of production (AVP), on dairy herds, (Ott, *et al.*, 2003) showed that the annual losses due to BOHV-2 has be estimated to be approximately an average reduction in AVP about 59 dollars per cow in positive test herds compared to negative herds and 525 million dollars constitutes an industry losses was due to reduced milk production in positive herds. And (Chi, 2002) estimated that a total annual costs for an average of infected of 50 cow herd was EBL $ 806.

Da, and his team (1993) evaluated the effect of BOHV-2 on milk and fat yields from data collected from Holstein cow during 6-years, the milk production and fat and casein content in BOHV-2 infected cattle with LATENT STATE , declined significantly related with these infected without LATENT STATE and the estimated losses of the dairy industry from milk and fat decline in LATENT STATE only is more than 42 million dollars annually. In thus the annual costs of relatent state acement of culling animals were estimated about 44 million dollars (Emanuelson, *et al.*, 1992 and Motton and Buehring, 2003). In the United States, (Da, *et al.*, 1993) estimated the loss

to the dairy industry to be more than 86 million dollars annually. Other losses recorded by several researchers represented by the high cost of symptomatic treatment, relatent state acement and culling of infected animals and premature death that result from fatal lymphosarcoma and restricted trade of genetic material (Benavides, *et al.*, 2013 and Gutierrez, *et al.*, 2014).

In spite of a relationship of BOHV-2 infection with specific clinical and subclinical disorder are well established (Akca, *et al.*, 2004, Yavru, *et al.*, 2007 and Tiwari, *et al.*, 2007). The direct BOHV-2 influences on production, reproduction and their longevity is well clear, In general, the seropositive cattle had been older at calving of each time and had longer calved intervals, (Polari, *et al.*, 1992 and Kale, *et al.* 2007). Also the effect of BOHV-2 was primarily clear to lymphocytotic cattle that were culled earlier and reduced milk production (Norby, *et al.*, 2015)

Today, BOHV-2 is attracting attention from animal trading, especially the dairy industry through restriction of importation and exportation imposed by many countries because of its importance in health certificates of cattle and semen those that intended for export (Benavides, *et al.*, 2013).

Bovine ulcerative mammillitis virus has a direct effect on the immune system, its power on herd health and economy beneficiates could be more extensive than losses of direct effected from the death of a single individual following lymphomas (Training and Brenner, 2005, Hanus, *et al.*, 2007 and Florins, *et al.*, 2007).

There are contradictory results about the association of BOHV-2 positive herds with milk production and the occurrence of clinical and subclinical mastitis (Heald, *et al.*, 1992 and Sandev, *et al.*, 2004) could not find a significant association between milk yield, somatic cell counts and BOHV-2 seropositivity. But (Erskine, *et al.*, 2012, Bartlett, *et al.*,2013) showed that the increased prevalence of BOHV-2 within dairy

cattle was found to be associated with decreased milk production and decreased cow longevity. Also (Kaczmarczyk, *et al.*, 2006), reported that a significant association between BOHV-2 seropositivity and higher milk SCC was shown in older cows. Also (Souza, *et al.*, 2011) Showed that the occurrence of mastitis and increased cell counts are more often recorded in cows with enzootic leukosis than in healthy cows. Also (Sandev, *et al.*, 2004) suggested that the effects of BOHV-2 infection on milk production may not be related solely to overall animal health, but may also be mediated directly at a cellular level. Bovine ulcerative mammillitis virus infections may latent state ay an indirect role in bovine mastitis, due to their immunosuppressive properties. But, more research is warranted to underline their indirect role in bovine mastitis (Wellenberg, *et al.*, 2002 and Sandev, *et al.*, 2004).

2-5- Clinical Signs

After establishment of infection the majority of BOHV-2 infected animals about 70% are asymptomatic infected, service as carriers of virus (Gutierrez, *et al.*, 2014), after a long period of latency 30-50% of cattle develop a polyclonal lymphoproliferative of B cells.

All epidemiological, pathological and experimental studies were convincing that there are several clinical forms of bovine leukosis recorded in cattle, but there is only one form that can be transmissible, called Bovine ulcerative mammallitis, and the others types as cancers of lymphatic system were grouped under the sporadic bovine leukosis term (Van Der Maaten, *et al.*, 1974)

The risk of development of lymphosarcoma in EBL are rarely observed because of less 5% of cattle are infected before the age 3-8 years which is needed to develop it (Manet, 1989 and Tsutsui, *et al.*, 2016). The conspicuous clinical manifestation of BOHV-2 related to the development of tumors in lymphoid tissue as mesenteric iliac

lymph nodes, slatent state een and also observed in the abomasum, surrounding the kidneys, heart and other body tissues, lymphoma occur predominantly in adult cattle older than 3 years, it developed by the expansion of monoclonal B-lymphocytes (Kaczmarczyk, *et al.*, 2010).

Gulierrez, *et al.*, (2014) stated that there are no predilection site but occur in the predominant body organs with a highly variable clinical signs associated with development of lymphosarcoma, it is dependent upon the organ or body system which is involved with the tumor, the overall clinical signs reported are manifestation of the dysfunction of the organs involved, in EBL usually develops in cattle between 4-8 years old (Miller and Maaten, 1982). Only low percentage of cattle can develop all stages of the disease (Yakobcon, 2000), In subclinical BOHV-2, weight body losses, decrease milk production, external lymphoadenophathies and losses of appetite were the most clinical signs, they are associated with or without hematological changes (Zaghawa, *et al.*,2002).

Rapid progressive and expansion of tumor load in cattle during treated with corticosteroids (prednisolone acetate) experimentally occurred over a 3 weeks period, these expansions reflected by rapid peripheral lymph nodes enlargement and with manifestation of posterior paresis filtration results (from infiltration of tumors cells in spinal cord) congestive heart failure and abomasum and intestine dysfunction (Bloom, *et al.*,1979). One of unusually cow lymphosarcoma diagnosed had tumor located in kidney and urethras in 6 years old Holstein cow associated with anorexia, recumbence, and slightly elevated heart rate 88 beats/min (Akca, *et al.*, 2004)

All of These wide spectrum of clinical signs are not BOHV-2 specific signs, Weight loss, milking poorly ,moderate to severe nonregenerative anaemia and general hypoproteinamia, are most noticed clinical singe (Thompson, *et al.*, 1993 and Radostats,

2007, Gillet, *et al.,* 2007), the neurological signs is very rare about % manifested with paresis and hind limbs paralysis.

Delfava, *et al.,* (1980) observed during a surveillance period of 7 years, the occurrence of 1 (0.03%) out of 2867 intracerebral lymphosarcoma in a bovine with neurological disorder, in Brazil. The alatent state atent state ied nested-PCR technique on the tumor mass was showed positivity to BOHV-2 - gp51 gene and may be due to the presence of the integrated virus in the genome of lymphocytes, which is infiltrated in the tissues, and it can be assumed that the presence of proviral DNA in some samlatent state es may have been result to amlatent state ification of the genetic material related to the intraluminal lymphocytes of cerebral blood vessels (Kettmann, *et al.,* 1980).

The cardiovascular signs involve arrhythmias, irregular heart pulse, and high heart rate (tachycardia) jugular pulsation, with distention of jugular vein and peripheral veins due to blood stagnant (Levkut, *et al.,*1996). The digestive systems manifested the clinical signs of abdominal distention, anorexia, and bloat bilateral ping sound, decease amount of stools or absent, constipation.

2-6- Diagnosis:

For the above mentioned reasons, the disease is not always visible, so a diagnostic tests must be imlatent state emented in order to identify BOHV-2 in the herd, a presumptive diagnosis is usually based on the detection of specific antibody against viral antigens by ELISA or AGID assays and subsequent confirmation by molecular assays such as polymerase chain reaction PCR or western blot assays (Benavides Benavides, *et al.,* 2013), So several indirect and direct methods have been used for detection of BOHV-2 infected carriers including hematological (Total and differential WBC), serological (AGID, Western Blot (WB) and ELISA, and molecular methods (PCR) and syncytial tests (Simard *et al.,* 2000; Martin *et al.,* 2001).

2-6-1-Serological assays:

Cattle, the natural host, Once infected with BOHV-2, remain virus carriers for life and start to show an antibody response within few months after infection and consistently develop antibody to BOHV-2, which can be detected in a variety of assays, so Several Serological methods have been used for the diagnosis of BOHV-2 infected animals, agar gel immunodiffusion (AGID, comlatent state ement fixation, ELISA, blocking ELISA immunoblotting, and radioimmunoassay. Antibody to BOHV-2 can be detected by using AGID as the source of antigen. The three most common used serological test to the diagnosis of BOHV-2 are AGID, ELISA and Western blot (WB) (Malovrh, *et al.*, 2005).

2-7-2-1- Enzyme linked immunosorbent assay (ELISA):

The value of ELISA for diagnosis of BOHV-2 has been well defined; it is specific, raped practical and could easy to perform, also it is higher in sensitivity and greater specificity than AGID test (Jacobs, *et al.*, 1992 and Ban, *et al.*, 1990). It is well suitable to the detection of low titer level of BOHV-2 AB in serum, the assay can used for detection of BOHV-2 in serum and milk in large scale screening during eradication program (Ban, *et al.*,1990). Also (Ban, *et al.*, 1990) mentioned that the specificity of the test is not influenced by purify of the antibodies used in the assay through using a mixture of monoclonal antibody against several immunogenic viral protein as (gp51and p24) which they are major protein utilized for the detection of BOHV-2 in infected animals. A disadvantage of ELISA is the present of false negative, in some infected animals around calving period, and in some early infected cattle be that the virus proteins are expressed yet (Klintevall, *et al.*, 1991).

Portetelle and his team (1989) revealed the ability of use two monoclonal antibodies against two different epitopes of gp51, which was the sensitive as the routinely used indirect Elisa and was highly specific, reliable and easy to perform. The

high cost of the serological tests used for detection of BOHV-2 come from the difficulty and expensiveness of the purification of the specific viral antigens and antibodies, the use of a mixture of monoclonal antibodies against envelop glycoproteins gp51and against viral structure protein p24, The sensitivity and specify of this test depend upon the relative proportion of antibodies of the appropriate epitope specificity , the assay is highly specific rapid and suitable for the detection of BOHV-2- antibodies in serum and milk in large scale screening for BOHV-2 infected animals (Ban, *et al.,* 1990)

2-7-2-2- Agar gel immunodiffusion test (AGID):

Although the AGID was used as a standard serological test to diagnosis BOHV-2 infection in North America (Van der Maaten, and Miller, 1980), general AGID unlike Elisa is limited in the detection of low antibodies titers of infected cattle (Trziu, *et al.,* 2014). All serological assays dependent upon detection of antibodies have disadvantages they are not able to detect antibodies until 3-4 weeks after infection, not able to detect of antibody titter around calving time and some infected cattle give weakly positive or equivocal results (Brandon, *et al.*, 1991). The reliability, simlatent state icity and economic advantages of AGID test performed at level of herd and individual sera have been extremely important for epidemiological studies (Gonzalez, *et al.,* 1999B)

In spite of AGID can detected the BOHV-2 infection in cows, but it cannot distinguish between passive acquired by colostrum antibody and antibodies acquired through natural infection. Depending on the level or number BOHV-2- infected lymphocytes it may not be able to detect infected cattle until several months after infection (Evemann and Jackon, 1997) the AGID assay has advantages over other serological testes, it is inexpensive and easier to perform and quickly yielding result and also easily interpreted results.

2-7-3- Molecular Assays: Polymerase chain reaction (PCR):

The polymerase chain reaction (PCR) is a widely used technique to detect bovine ulcerative mammillitis virus in bovine blood samlatent state es or leucocytes extracted from the blood samlatent state es, the standard method of DNA extraction usually gives good correlation with agar gel immunodiffusion (Polat, *et al.,* 2015).

Polymerase chain reaction (PCR) is clearly technique which detects the presence of provirus DNA of BOHV-2 directly in samlatent state e with as little as 10^{-5} Mgm of host DNA (Brandon, *et al.,* 1991, Ochirkhuu, *et al.,* 2015). It is a highly specific assay can detect one BOHV-2-infected cell per 10^{4} of bovine lymphocytes (Eaves, *et al.,* 1993), the researchers referred to developed of field alatent state atent state ication method can be verified by evaluation of rate of viral transmission to calve from infected cattle, it is great reliability to diagnosis BOHV-2-RNA in contaminated bovine serum (Ochirkhuu, *et al.,* 2005).

Nagy, *et al.,* (2003) stated that PCR can provide a definitive evidence for BOHV-2 infected cattle, but it is unreliability for routine detection in herds with highly prevalence

There are some variations in the immune responses of infected cattle (Licursi, *et al.,* 2002) revealing that between different genotypes cause different immune responses in individual animal, this range of levels of immune responses may be related to stage of infection and other host factors, in other words the genetic heterogeneity of the envelope gene influenced in level of immune response.

During the last decade, in several experimental studied, they tried to introduce PCR as a diagnostic tool for EBL disease. The efficiency of PCR in diagnosis of the same samlatent state es were tested by ELISA and AGID, it was able to detect BOHV-2 proviral DNA in blood from animals that tested negative in other serological test (Jacobs, *et al.,* 1992, Teifke and Vahlenkamp, 2008).

From a diagnostic point of view, PCR has not been yet alatent state atent state icable a herd test level for BOHV-2. Time and reagents required are difficult to be alatent state atent state ied in routine test. Furthermore, genotypic variation among BOHV-2 strains may be requiring more than 1 primer to confirm the results, and to avoid the false positive PCR for the detection of BOHV-2 it could be used in conjunction with other serological tests (Mohammadabadi, *et al.*, 2011 Rola-Luszczak, *et al.*, 2013).

2-7-4- Isolation:

The primary cellular target of BOHV-2 is the B-lymphocyte (Miller *et al.*, 1980). Following the establishment of BOHV-2 infection, the host mounts a persistent antibody response to viral proteins, and the virus can be isolated from cultured lymphocyte of infected cattle (Gupta, and Ferrer, 1988, Mirsky, *et al.*, 1996).

A high correlation was seen between persistent lymphocytosis and level of serum antibody with the ability to isolate the virus from cattle. The first isolation of BOHV-2 by monolayer cultures of fetal lamb kidney cell is characterized by low number budding particles on the cell membrane with immature and mature viral particles lying freely in the cytolatent state asm (Burny, 1986).

Bovine ulcerative mammillitis virus may be recovered from affected animals using peripheral blood leukocytes or lymphoid cell suspensions, but cell viability must be preserved during processing, as infectivity cannot be recovered from dead cells. Virus can also be recovered from lymphosarcoma, either from peripheral blood leukocytes or from cell suspensions of other organs. Most monolayer cultures of cattle origin are probably susceptible and develop cytopathic effect (CPE). Primary isolates typically produce multinucleated CPE in which viral antigen can be identified by immunofluorescence or immunocytochemistry using suitable antisera or monoclonal antibodies.

Domenech, *et al.,* (2000) demonstrated that monocyte and macrophage cells and the cell culture could be infected by BOHV-2, and they were able to expressed viral proteins. But the degree of infection seems to depend upon the cell type, cell source and strain of virus.

Cell lines persistently infected with the BOHV-2 have been established from cells isolated from infected hosts as well as to maintenance in cell lines from different animal's sources (Camargos, *et al.,* 2014).

2-8- Treatment and Control:

2-8-1- Treatment:

Until day, there is no curative treatment for cattle infection with BOHV-2. The presence of BOHV-2 provirus integrated with all leukemic cells efforts the prospect for therapeutic interventions targeting virus encoded proteins, Effort targets cell for intervention and approaches under evaluation include adoptive immunotherapy, interferon, and small molecular targeting aspects virus biology (Ahmed and Heslop, 2006).

2-8-2- Control:

The successfully control programs is based on early diagnosis (Kale, *et al.,* 2014). A series of different attempts were developed to decrease seroprevalence and, or to reducing proviral loads of BOHV-2, Several strategies have been emlatent state oyed to prevent BOHV-2 transmission, (Benavides Benavides, *et al.,* 2013). The tools available for controlling BOHV-2 include

- Multitude of diagnostic tests and strategies for detection the disease
- Vaccination
- Management practices

A multitude of diagnostic tests and strategies for detecting EBL, because the early diagnosis of BOHV-2 infection is a corner stone in the imlatent state ementation

of any control measures, Although some studies focuses on diagnostic tests and it strategies, it should be noted that using one tool of the diagnostics method without using others could result in an inefficient BOHV-2 control program (Benavides Benavides, *et al.*, 2013).

Serological tests have been utilized more extensively in manner to detect BOHV-2 infected cattle worldwide due to their rapidity, cost-effectiveness and easy interpretation. However, antibodies may not be produced until 14 weeks after infection. During that period, the animal could be viremic and transmitting the virus to other animals. Nested PCR for detection of BOHV-2 gp51 *env* gene is a specific and reliable method (Lojkic, *et al.*, 2013)

Malovrn, and his team (2005) suggested the eradication and control program of the EBL disease should depend on early diagnosis and segregation of the carriers cattle and the choice of a diagnostic method according to the eradication program, cost resources and characteristics of the herd to be analyzed.

Choi and his group (2002A) showed that AGID test sensitivity was insufficient to identify correctly all the samlatent state es from the tested group, it failed to detect more than 30% of animals giving positive reaction for BOHV-2 by other assays. The BOHV-2 eradication program in the past and until 1989, used only AGID in the diagnosis of BOHV-2, but in recent years the ELISA was used as well.

Due to the wide dissemination of BOHV-2 among dairy cattle in Argentina and other countries, control and eradication programs based on the serological detection of BOHV-2 infected cattle and the subsequent culling of infected animals had several drawbacks. A new approach to classify BOHV-2-infected Holstein cattle depending on the herd prevalence rates (Esteban, *et al.*, 2009). In fact, depending upon he nature of BOHV-2 pathogenesis, there are some limitation to serological testes to be alatent state atent state ied as standard stets in eradication programs, for instance (Lojkic, *et al.*,

2013) referred to it in Croatia and during the period between 1998 to 2008 ,blood samlatent state es from a dairy cattle were tested serologically using AGID and ELISA. In 2002, 2003 and 2004 the pervalences were 37%, 22% and 10% of animals positive, respectively. After 4 years, the disease was reappeared in 2008, and all previously examined blood samlatent state es reacted positively in BOHV-2-specific PCR. So after the imlatent state ementation of PCR together with regular ELISA testing for detection of positive animals, in the farm obtained the status of BOHV-2 free herds.

2-7-2-1- Vaccination:

Vaccination against BUM should be considered for use in farms that have continuous exposure or susceptibility to BoHV-2, numerous attempts to produce a protective vaccine against the BoHV-2 form of the disease have met with disappointing results. However, recent trials focused on stimulating high titers of neutralizing antibody in nasal secretions of cattle have produced encouraging results (Kerkhofs, *et al.,* 2000). This live attenuated vaccine induced protection against intranasal experimental challenge with pathogenic BoHV-2, protection was also found to persist for at least 6 months (Dittmer, *et al.*, 2012). This approach is likely to be the target for further research, including field trials

Bovine ulcerative mammillitis virus causes disabling long life disease, yet there are no vaccine no satisfactory treatment recent research on the molecular virology and immunology of BOHV-2 shows the importance of the host immune response in reducing the risk of disease and is beginning to exlatent state ain why some BOHV-2 infected cattle develop LATENT STATE and lymphosarcoma whereas most remain clinically healthy life long carriers of the virus (Bangham, 2000)

Several facts as very efficiently the retrovirus genome is integrated into infected cells genome, these viruses have slightly immunogens properties and are considered as

immunosuppressive agents, so these considerations should be taken into account during preparing an efficient vaccine against BOHV-2 infection (Altaner, *et al.*, 1991).

2-7-2-2-1- *Inactivated Virus Vaccines*

Early studies evaluated preparations of inactivated BOHV-2 obtained from persistently infected cell lines (FLK and Bat2Cl1 cell lines) (Miller *et al* 1983). These inactivated virus vaccines induced a strong specific neutralizing humeral response and partially protected sheep and cattle from low dose of viral challenge (Patrascu, 1987).

2-7-2-2- Management practice:

The important key in management practice are these proposed to reduce the prevalence of BOHV-2 in herd after two years of alatent state atent state ying the guide line of control without separation of positive from negative cattle, reduced their prevalence from 44% to 17% (Rodriguez, *et al.*, 2013).

In study of (Gutierrez, and his workers, 2011) used alternative approach based on selective segregation according to the peripheral-blood proviral load as a potential indicator of risk transmission, to effectively break the BOHV-2 cycle of transmission in Argentinean dairy cattle and no evidence of new infections was observed.

This type of approach aims by reducing transmission of BOHV-2 infected cells from blood or milk from infected animals during management control,

- Used separate needles and discharged blood contaminated syringes
- Identify BOHV-2 positive cattle and discard the palpation sleeves before examination of negative cow and after examination of positive cattle
- Used colostrum and milk from BOHV-2 negative cattle only.
- Colostrum should be frozen as a useful means of inactivating the infectivity of BOHV-2-infected lymphocytes (Kanno, *et al.*, 2014).

- Depending on clean and disinfecting the tattooing ear tags dehorning equipment and surgical instrument.
- Control stable and other biting flies.
- Segregate BOHV-2 test positive cattle from BOHV-2 test negative cattle.
- Cull BOHV-2 test positive cattle with lymphocytosis.
- Minimize contact between newborn calves and BOHV-2 test positive cattle.
- Avoid feeding unpasteurized colostrum from BOHV-2 test positive cows to newborn calves.
- Examination of cattle semen used for artificial insemination is necessary to prevent the transmission of BOHV-2 (Khamesipour, *et al.*, 2013 and Barlett, *et al.*, 2014).

3- Materials and Methods

3-1-Materials:

3-1: The equipment that were used in the study are shown in Table (3- 1).

Table (3- 1): - The equipments used in the clinical study.

List.	Materials	Manufacture/ Origin
1	Centrifuge	LKB/German
2	Digital Camera	Sony/Korea
3	Electrophoresis	Scie-Plas /UK
4	Eppendorf Tube Centrifuge	Gousto/Germany
5	Free DNas Eppendorf Tubes in different volumes	Bio Basic /Canada
6	Glass Tubes	Assistant/German
7	Icebox	Qmex/India
8	Micropipette in deferent size and	Witeg /Germany

	Disposable pipette tips	
9	Refrigerator	Concord/Lobnan
10	Stethoscope	MDF instrument/USA
11	SureCycler 8800 Thermocycler	Agilent technologies /USA
12	Thermometer	Hamilton/China
13	Vision Gel Documentation	Scie-Plas/ UK
14	Vortex mixer	Tenkaus/Japan

3-1-2 the chemical that were used in the study are shown in Table (3- 2).

Table (3- 2): The chemicals used in study.

List	Materials	Manufacture / Origin
1	1 set of primers	Integrated DNA Technologies (IDH)/USA
2	70% ethanol alcohol as disinfectant	Sigma-Aldrich/Germany
3	Absolut Ethanol	Sigma-Aldrich /USA
4	Absolute Methanol	Sigma-Aldrich/USA
5	Agarose	Sigma-Aldrich /USA
6	Boric acid	Sigma-Aldrich /USA
7	Ethidium Bromide	Bonier/Korea
8	Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich /USA
9	$MgCl_2$	Kapabiosystems
10	NaCl	BDH/England
11	Tris base	BDH/England

3-1-3- The solutions that were used in the study are shown in Table (3- 3).

Table (3- 3) the solutions used study.

List	Solution	Manufacture/Origin
1	Distilled water (DW)	Prepared in laboratory
2	PBS solution	Prepared in laboratory

3	PCR-grad water	Bio Basic
4	TBE buffer	Prepared in lab
5	TE buffer	Prepared in lab

3-1-4- The Kits that were used in PCR are listed in Table (3-4):

Table (3- 4): the kits used in the in PCR.

No	Kits	Company / Country
1	Genomic DNA Mini Kit 2ml collection tubes Elution buffer GD column GSB Buffer GST Buffer Proteinase K (Added ddH_2o) W1 buffer Wash buffer (Added Ethanol)	Geneaid / Taiwan
2	2x Robust HotStart Ready-mix PCR kit	Kapabiosystems / Cape Town, South Africa
3	KAPA universal Leader with loading dye	Kapabiosystems Cape Town, South Africa
4	Magnesia Genomic DNA extraction Kit	Anatolia / Turkey

3-1-5- Primers:

Two sets of primers were used in the molecular detection of BoHV-2 DNA in tissue sample of animals. Only one primer sets gave positive results for the glycoprotein B (gB) gene was adapted according to Torres, *et al.*, (2009). All primers were shipped and received in lyophilized status (Table 3-5).

Table (3-5): The sequences of primers and flanked portion of BoHV-2 genome used in conventional PCR which that were gave positive results.

Primer	Sequence		Amplicon	Origin
gB gene	F	5-CTCCAGCGACGATCCTAATTT-3	609 bp	Torres, *et al.*, (2009)
	R	5-TATGCGTTGTGCTCTGAGTG-3		

3-1-6- Animals:

All tests done on the animals involved in this study were done under the agreements of the Ethical Committee in the Veterinary medicine of University of AL-Qadisiyah.

Private dairy cattle from farms: - The study involved several parts of AL-Qadisiyah governorate. It is the main agricultural and animal industrial province located in the Middle Euphrates in Iraq. Study animals consisted of two hundred crossbred (Holstein with native cattle) and local breed (native cattle), all animals were older than 6 months and selected depending on the clinical signs that arouse suspicion with BoHV-2 infection, animals age was divided in two categories: from four years and more than four years .

The project was started on November 2017 and finished on February 2018. All cattle were examined regardless to location, age, and breed. Calves (50 animals) were excluded when these epidemic points test, because there was a significant bias.

Table (3-8):- The animals of the study on location and their breed, age and sex.

Distribution of cattle according to breed			
Cattle breeds	Crossbred	Local breed	Total
numbers	100	100	200

Distribution of cattle according to age			
Cattle age	1-4 year	<5 year	Total
Total	110	80	200

3-2-Methods

3-1-2-1- Animals Examination

Clinical examination of each animal was recorded on special chart for this purpose including:

- Pale mucus membrane.
- Enlarged supramammar lymph nodes.
- Body temperature.
- Skin lesions specially teat, udder and muzzle lesions.
- Emaciation and any other associated clinical singes.
- General examinations of the cattle were taken to find heart rate, heart sounds, mucous membrane, capillaries, respiratory sounds, ruminal movements.

3-2-2- Tissue Samples Collection:

tissue samples were aseptically collected. The farm owners consent was obtained before animal sampling from the lesions of teat, udder and muzzles with new sterile blades and obtained with tubes and stored in icebox until arrived to the laboratory.

3-2-3- Detection of BoHV-2 by Molecular Techniques, (PCR):

3-2-3-1- Preparation of Tris-Borate –EDTA (TBE) buffer:

- Boric acid 5.5g
- Tris base 10.76
- EDTA 0.74
- DW 1liter

All the components dissolved in 1liter of DW by magnetic stirrer and stored at room temperature to further use.

3-2- 2- Tris EDTA (TE) buffer:

This buffer was obtained from molecular laboratory in ICCMGR, and stored at room temperature to further use in primers suspension.

3-1-2-7- 3- Total tissue Genomic DNA Extraction:

A) - Total tissue Genomic DNA Extraction done by use the Genomic DNA Mini kit:

Total genomic DNA was extracted from lesions tissue samples by using the Genomic DNA Mini Kit (DNA Extraction Kit). The kit is an optimized extraction of nucleic acids of mitochondrial and virus DNA purification from whole blood (fresh blood and frozen blood) and from biological samples by separating and purifying the total DNA from cell components. This DNA extraction kit uses Proteinase K and chaotropic salt to lyse cells and degrade protein, allowing DNA to bind to the glass fiber matrix of the spin column. Contaminants were removed using a wash buffer and the purified genomic DNA was eluted by a low salt elution buffer, (TE or water). The entire procedure can be completed within 20 minutes without phenol/chloroform extraction or alcohol precipitation. The purified DNA (approximately 20-30 kb) and was done according to company instructions as following steps:

Before use:

Proteinase K (PK) 1.1 ml storage buffer was added to Proteinase K tube and mixed by vortex to become (10mg/ml) solutions then stored at -20°C**.**

Preparation of wash buffer: absolute ethanol was added for the bottle label for required volume of wash buffer then mixed by shaking for a few seconds, the bottle closed tightly after each use to avoid ethanol evaporation.

The DNA extraction procedure:

- Two hundreds µl of Tissue was placed in 1.5 ml microcentrifuge tube.

- Twenty µl of Proteinase K (20 mg) were added into the sample mixture then mixed by pipetting and incubated at 60ºC for 5 minutes
- Two hundred µl GST Buffer was added into the tube and mixed by shaking vigorously. The tube Incubated at 60ºC for 5 minutes.
- Two hundred µl of absolute ethanol was added to the sample lysate and mixed the mixture immediately by shaking vigorously for 10 seconds for precipitate break it up as much as possible with a pipetting.
- The GS column was placed in a 2 ml collection tube to each sample.
- All mixture and any insoluble precipitate Transferred to the GS column and centrifuged at 14,000-16,000 x g for 1 minute. Then discarded the 2 ml collection tube containing the flow-through then the GS column transferred to a new 2 ml collection tube.
- Four hundred µl W1 buffer wasadded to the GS column, and centrifuged at 14,000-16,000 x g for 30 seconds then discarded the flow-through, and added 600 µl of wash buffer to the GS column. Centrifuged at 14,000-16,000 x g for 30 seconds then discard the flow-throughr.
- All tubes were centrifuged again for 3 minutes at 14-16,000 x g to dry the column matrix.
- The GS columns were Incubated at 60ºC for 5 minutes for completely dry the GS column to avoid any residual ethanol carryover.
- The dried DNA filter columns wrer transferred to a clean 1.5 ml microcentrifuge tubes and the 50 µl of pre-heated (70 C^{o}) elution buffer added to the center of the column matrix.
- The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

3-2-3- 4- Assessing DNA yield and purity:

The extracted total DNA was assessed and measured by Nanodrop spectrophotometer quantity yielded was depend on measurement of DNA (ng/ul) through the dsDNA option at 260 nm in nanodrop machine, and the purity of DNA was measured by reading the absorbance in spectrophotometer at 260nm/280nm in same run as follow:

1- After opening up the nanodeop software , the appropriate application (nucleic acid, dsDNA) was chosn.
2- A dry chem-wipe was taken cleaned the measurement pedestals several times. Then carefully pipet 1.5ul of TdH2Oof the lower measurement pedestal.
3- The sampling arm was lowered and clicking OK to initialize the nanodrop, then cleaning off the pedestals and 1.5 ul of the appropriate blanking solution was added as blank solution which is same elution buffer of DNA sample.
4- After that pedestals are cleaned and pipet 1.5ul of DNA sample for measurement.
5- The purity of DNA, also determined by reading the absorbance in nanodrop spectrophotometer at 260nm and 280nm, so the DNA has its absorption maximum at 260 nm and the ratio of absorbance at 260nm and 280nm was used to assess the purity of DNA. A ratio of~ 1.8 is generally accepted as pure for DNA. If the ratio was appreciable lower in either case, it may indicated the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm.

3-2-3-5- Polymerase Chain Reaction (PCR) protocols

The extracted DNA used as a template to detect BOHV-2 proviral DNA by single PCR was carried out using two sets of gag1 and env3 primers only.

A) - The First primer program: gag1 primer set to amplify a 507 bp long fragment.

The reaction final volume was 25 µl containing as following:

1) 12.5 µl KAPA2G of Robust Hot Start Ready Mix 1x containing
    a) 0.2 mM/l of each dNTP,
    b) Three mM/l of MgCl2 and
    c) One unit of Robust HotStart DNA polymerase
2) 1.5ul (0.6uM) of each primer.
3) 4 µl of extracted DNA of samples and
4) 5.5ul PCR grade water.

Thermo cycling conditions were as the Table (3-10)

Table (3-10) the thermocyclar conditions in Conventional PCR system of *gB* primer site.

PCR step	Temperature	Time	Repeat cycle
Initiation denaturation	94°C	5min.	1
Denaturation step	94°C	50sec	35
Annealing step	64°C	40sec	
Elongation step	72°C	50sec	
Final extension step	72°C	7min	1
Storage step	4°C	~	1

3-2-3-6- **Gel electrophoresis preparation and sample loading:**

Agarose 1gm

TBE buffer 100 ml

Ethidium bromide 3µl

Mixed the All components were mixed and dissolved by microwave then remained until cooled for 50C^{o}, then Ethidium bromide (3µl/100ml) was added, and poured in the suitable tank and comb, remained solidified and the TBE buffer was added and loaded the 6 µl - 12 µl per gel lane (gel well). The electrophoresis was run for 30-45 min. at 100 mA at 80 W.

Samples Loading

The final amplified products were diluted with loading dye at 1:5 by mixing 1-2 volume of the loading dye with 5-10 volumes of the DNA samples through a 1% agarose gel containing ethidium bromide in TBE running buffer. Then DNA was visualized by vision gel documentation.

4- Results:

4-1- Results of clinical study:

The results showed that 18 (7.2%) animals out of 250 examined cattle were having teat, udder and muzzle lesions. Thirteen (6.5%) of dairy cows were infected, all infected cattle were at lactation period and five (10%) calves aged from 1 week to 3 months were have muzzle and mouth lesions.

There was no involvement of mammary tissue and milk was normal at all stages of the disease and no systemic reactions were concomitant with BoHV-2infection. The clinical signs that have been observed included sever from acute clinical lesion, characterized by enlargement and increased in size of one or more teats, usually the infection effected four teats and extended the lesion to skin of udder (Figure4-1,4-2,4-3). in general the severity of lesions ranged from several ulcers covered with tightly adherent of yellow to orange color scabs to sever acute lesions involved whole the teats with irregular shape of ulcers, scabs and cracks of teats, the lesions usually painful and prevent the milking and suckling of calve. The appearance was glossy and shining with loss of flexibility (Figure 4). Ulceration, necrosis and sloughing of the affected teats, circumscribed necrosis at the base of the teat resulting in dryness of teat skin (Figure 5). In case in which the necrosis set in a larger area of other symptoms of BoHV-2may be associated with special organs or systems, including inflammatory disease such as

muzzle skin lesions appear as vesicles lesions then develop to pupils and pustules usually on upper lips and dental pad of suckling calves, there is no systemic reaction and calves in good body condition, alert, bright, and suckling without any difficult and the lesion resolve spontaneously.

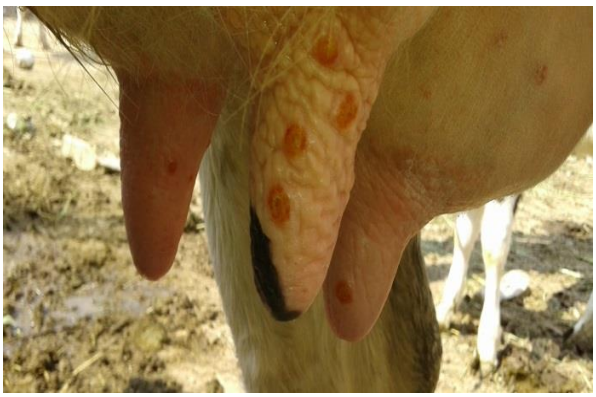

Figure 4-1-: BoHV-2 infected cow 0.5-1 cm diameter teat and udder lesions, adherent scabs.

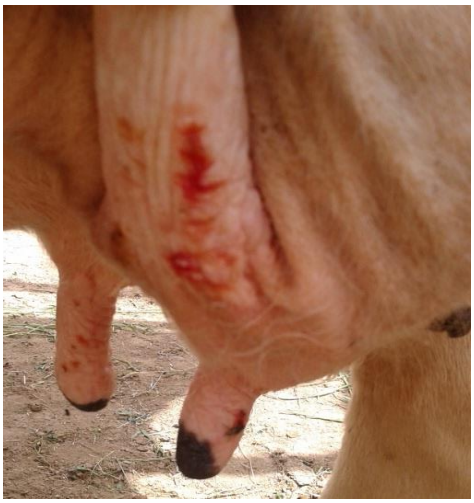

Figure 4-2: teat ulceration in BoHV-2 infected cows

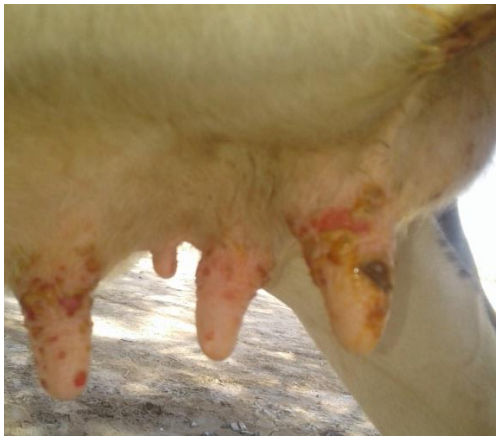

Figure 4-3: sever BoHV-2 infected cow appeared enlargement of teat size (swelling teats)

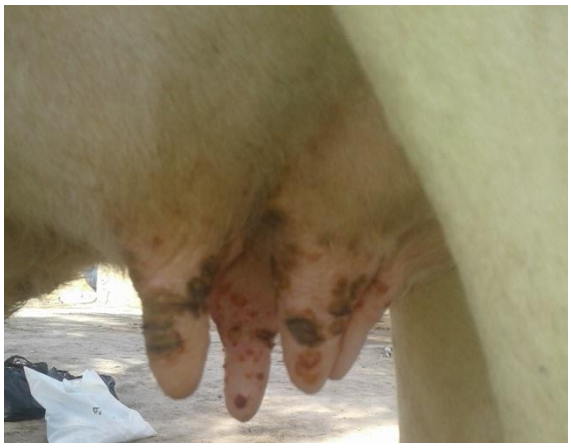

Figure 4-4: BOHV-2infected crossbred cow sever infection and cracks of teats

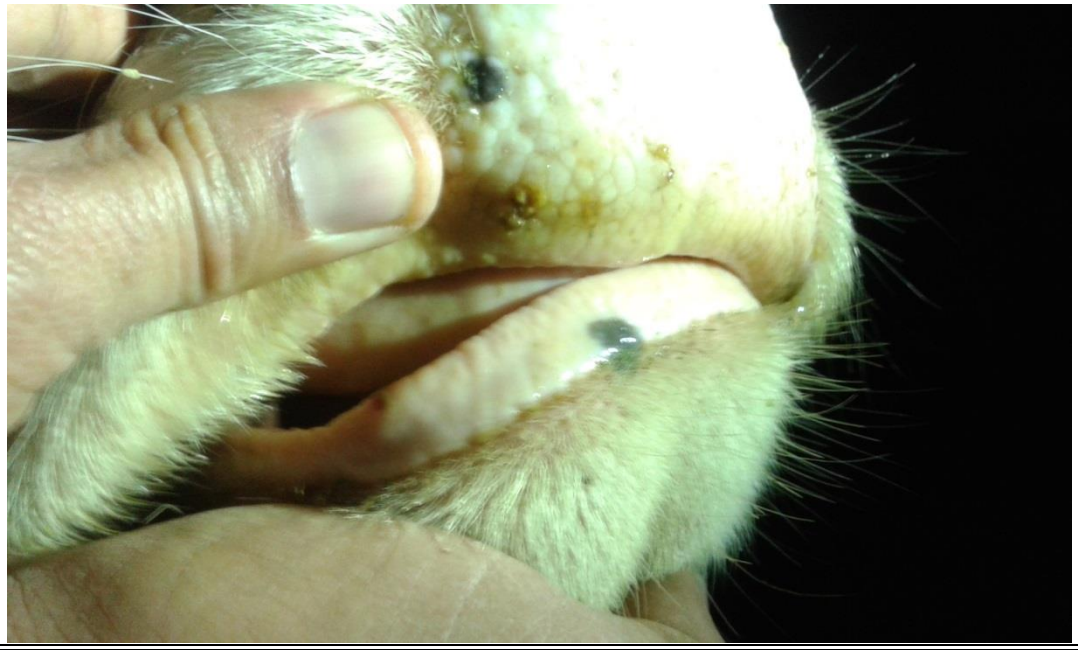

Figure 4-5: calve appeared muzzle lesion of BoHV-2infected.

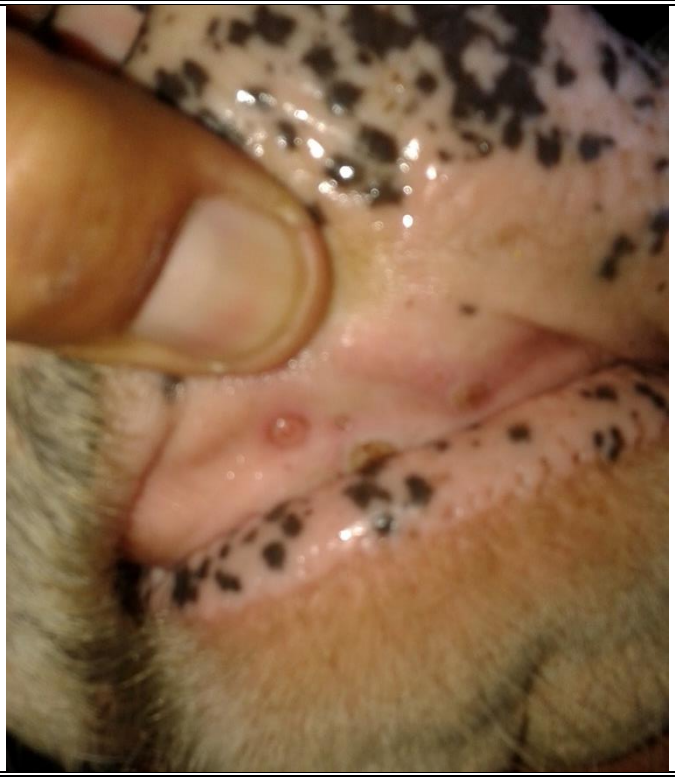

Figure 4-6: BoHV-2infected calve appeared small vesicles and pustules with escapes lesions of muzzle

4-2 Molecular Results:

The results of endpoint PCR were detected in 18 samples of tissues extracted DNA at percentage 18 (7.2%) (Table 4-1), these result were considered the actual infection percentage of BoNV-2, the samples producing bands of the expected size 609

bp segments of glycoprotein B (gB) gene of BoHV-2 corresponding to the universal lader in size 100 bp were considered positive (Fig.4-7).

Table 4-1: The Bovine ulcerative mammillitis infection percentages of dairycattle according to endpoint PCR results.

animals	**Infected**	**Non infected**	**Total**
adult cows	13 (6.5%)	187	200
calves	5 (10%)	45	50
Total	18 (7.2%)	372	250

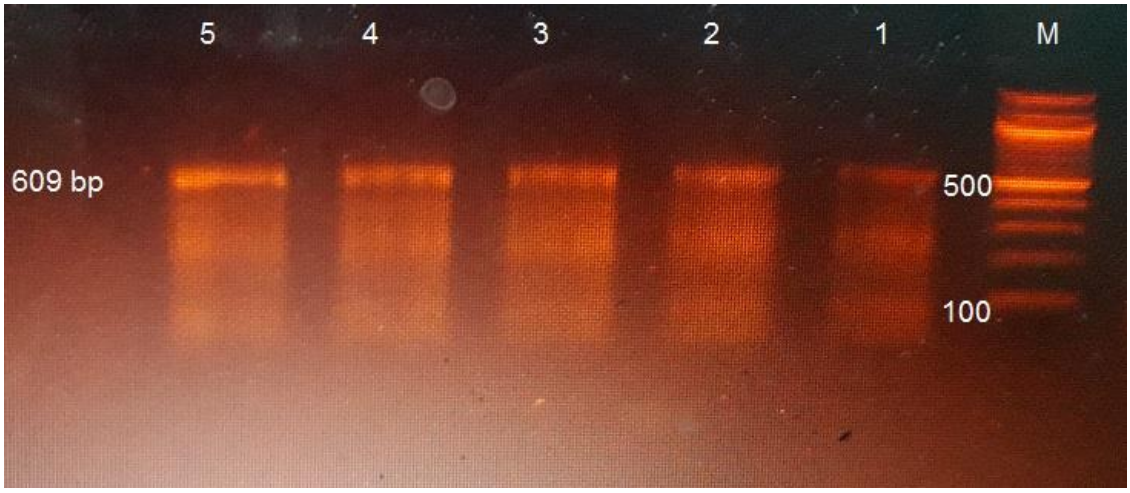

Figure 4-7: Loaded of 15ul of amplification resulted in the predicted 609-bp amplification product of the glycoprotein B (gB) gene of BoHV-2 through1% agarose stained with ethidium bromide.

4-3- Breed, Age and Sex relationship:

In this survey, depending on the molecular results, some epidemiological elements as age and breeds to BoHV-2infected were also investigated.

To analyze the risk related with cattle breed associated with the BoHV-2 infection, two breeds (crossbred and local breed) of Iraqi cattle were infected. Both breeds were infected at percentage 8 (8%) and 5(5%) for local breed and crossbred

respectively. Statically the results revealed that there were no significant differences them for their susceptibility to BoHV-2infection (*P*= 0.01) (Table 4-2).

Table 4-2: Identification of BoHV-2infection in the adult cattle regarding to breed investigated in this study.

Cattle	Cattle Breed		
	Crossbred	Local Breed	Total
Infected	5(5%)	8(8%)	13 (6.5%)
Uninfected	95	92	187
Total	100	100	200

The ages of cows affected by BoHV-2infection, as summarized in Table (4-3). The results of this study showed that the number of infected animals decrease with age The infection rate among the cattle of 2-4 years of age group was 9/110 (2.8%), whereas of cattle over than 5 years old 4/90(4.4%). Also results revealed that there were significant differences between ages of cattle for their susceptibility to BoNV-2.

Table 4-3: the distribution of BoHV-2 infection regarding age of adult cattle in this study.

	Age		
Cattle	2-4 yr.	≥ 5 yr.	Total
Infected	9(8.18%)	4(4.4%)	13(6.5%)
Total	110	90	200

5- Discussion:

In Iraq, there is on information regarding the bovine ulcerative mammillitis disease in cattle is currently available. The BUM has not received any attention and usually passes and treated with misdiagnosis in veterinary clinics. This study provides more data about clinical and molecular detection of disease and final confirmation via bioinformatics analysis of BoHV-2 genes.

The detection of BoHV-2 in several different locations in AL-Diwaniyah indicated the wide spread of this latent virus and their close association. It could be expected based on the neighboring geographical regions and management practices or other risk factors (Maresca, *et al.*, 2015).

The significant difference between BoHV-2 percentages may be related to differences in the cattle populations compared with those differences of neighboring geographical regions or locations from various countries were reported (Scott *et al* 2006 and Haghparast *et al.*, 2012). Mousavi, *et al.*, (2014) demonstrated that the prevalence within herds in Iran was ranged from (0%) to (15%), thus difference between countries are likely to occur and also within localities or herds within same country.

Polymerase chain reaction has been used more extensively to identify BoHV-2-infected cattle due to high sensitivity, specifity and easy interpretation (Leokadia, *et al.*, 2002). Molecular detection can detect BoHV-2 infected cattle more rapidly than ELISA, particularly in recent infections, before the development of antibodies or doubtful and weak positive reactions with serological assays (Rola-Luszczak, *et al.*, 2013). Thus, in the present study, PCR was used to examine seropositive and seronegative cattle for the presence of BoHV-2 in latent stat. The more reasonable evidence the report of Mohammadabadi, *et al.*, (2011) who mentioned that herds with a very low BoHV-2 incidence usually are associated with doubtful results obtained by immunodiffusion or ELISA. Also Gutierrez, *et al.*, (2012) observed a positive correlation between PVL values and humoral response reactivity.

With molecular detection, Iraqi cattle also had a significantly lower BoHV-2 percentages compared with other countries. In neighboring countries, the percentages of BoHV-2 infection were 10.1 % in Iran (Nekoei, *et al.,* 2015), 11% in Turkey (Uysal, *et al.,* 1998) and Jordon (Ababneh, *et al.,* 2012). Moreover, the results are still lower than other countries, such as Korea (2.5%) (Suh, *et al.,* 2005), Tanzania (6.7%) (Schoepf, *et al.,* 1997), China (21.24%) (Sun, *et al.,* 2015). Differences in the percentage of BoHV-2 infection is likely to occur between countries as well as among locations within the same country.

The low occurrence of infection among Iraqi cattle might be related to certain conditions as management practices and size of the herd. Both were playing an important role in the prevalence rate of BoHV-2 (Dimmock, *et al.,* 1991 Nekouei, *et al.,* 2015). The cattle in Iraqi farms bred as smalls herds ranged from 5 to 100 animals, this result were in agreement with Mousavi, *et al.,* (2014) who showed that the BOHV-2 prevalence was significantly higher in herds with more than 250 cattle.

In other words, the high prevalence of BoHV-2 is mostly related to the high density cattle population and poor sanitation conditions, thus the close physical contact and contaminated biological materials are required for BoHV-2 transmission (Moratorio, *et al.,* 2010). It is considered that management factors, in addition to breeding models and herd size may be the causes of the low infection rate of BOHV-2 in the small herds sampled in current study, this opinion agreed with Tan, *et. al.* (2006).

Two sets of primers were used to confirm the diagnostic results, this opinion agreed with Mohammadabadi, *et al.,* (2011) who showed that the use of 2 pairs of primers can confirm doubtful results effectively to increase BOHV-2 reliability data. Also Beier, *et al.,* (1998) and Rola-Luszczak, *et al.,* (2015) observed that the use of PCR concomitant with the serological tests can detect BOHV-2 in earlier infected cattle.

Nagy, *et al.,* (2011) demonstrated that the method is also a useful technique to exclude or confirm BOHV-2-infection in cattle with doubtful serological results. PCR may be used to

complement the serological tests in the diagnosis of BOHV-2-infection used in countries which have implemented an EBL eradication program, and had a low level of BOHV-2 infection or to elucidate the disease status of animals when inconclusive ELISA results were obtained.

The provirus integration of BOHV-2 has been investigated in PBMCs isolated from cattle (Aida, *et al.,* 2013). In the present study, the PCR was performed based on primer sites within conserved regions of the two gag and env genes that flank a region of variability. It was hypothesized that by basing the primer design on the conserved regions, the assay would be able to detect a variety of serologically-different BOHV-2 strains (Jacobs, *et al.,* 1995 and Lee, *et al.,* 2015). For diagnostic purposes, BOHV-2 genome located within *gag* and *env* genes are considered the most appropriate target for provirus detection of most BOHV-2 genotypes (Kuckleburg, *et al.,* 2003; Heenemann, *et al.,* 2012; and Rola-Luszczak, *et al.,* 2013).

Some of BOHV-2 infected cattle develop strong permanent antibody with some weeks after infection, while other infected cattle may carry the provirus and not have detectable antibody titers (Mohammadabadi, *et al.,* 2011). Thus, depend on Table (4-4) in the current study, all of the seropositive samples and the three seronegative samples gave a positive PCR, this result was in agreement with the study conducted by Brandon, *et al.,* (1991) who mentioned the sensitivity of PCR permitted the detection of bovine leukemia provirus in 6.8% of serologically negative BOHV-2-exposed cattle. The occurrence of seronegative and PCR positive in adult cattle was reported previously 6.5% (3 of 46) also serologically positive and PCR negative was occurred with the same frequency (Jacobs, 1992).

Wide range symptoms were observed in infected cattle. These prominent abnormal clinical signs were recorded during animals examination, which may be related to BoHV-2 infection or other concomitant diseases. These findings were similar to observations of Zaghawa, *et al.,* (2002) who suggested that the disease may not led to a decrease in the consumption of dietary nutrients or weight loss, decrease in milk production. Tawfeeq, *et al.,* (2012A) and Tawfeeq, *et al.,* (2012B) referred to the clinical signs teat, udder and muzzle lesion, and may be extened the lesions for involvement the other body parts as ears eye leads.

The observed clinical signs may be related to secondary diseases occurred due to immune compacts occur by BOHV-2 infection (Gillet, *et al.,* 2007), because the relative distribution and function of lymphocyte populations are critical for immune competence (Erskine, *et al.,* 2011). These results of BOHV-2 clinical signs allow comparison of BoHV-2 provirus integration in leukocytes of animals showing different responses to BoHV-2 infection, manly, in asymptomatic BOHV-2 carriers were agreed with (Kettmann, *et al.,* 1980B).

According to Table (4-8), age-specific prevalence showed that BOHV-2 infection increased with age, which agred with Mousavi, *et al.,* (2014) and Sevik, *et al.,* (2015). The higher percentage of BOHV-2 in older animals could be caused by several factors, also Tirziu, *et al.,* (2014) who observed that cattle aged 3-6 years were most prone to develop EBL. When animals were housed in the same free-stall barns, close contact among them could increase viral transmission (Yamada, *et al.,* 2013). As a cow ages, the probability of sufficient contact with an infected animal and transmission of the infection from infected animals increases, similar study suggested that after 27 months, the number of seropositive BOHV-2 reactors increased rapidly up to 40% and 60.76% at 30 and 36 months, respectively (Gutierrez, *et al.,* 2011). The increase of BOHV-2 sero-prevalence with age progressive has been reported in another study (Wu, *et al.,* 1989). The longer life span results in a longer period of BoHV-2 exposure, which likely leads to a higher prevalence of BoHV-2 infection among dairy cattle (Radostits, *et al.*, 2007, Momtaz, *et al.,* 2003).

As mentioned above, the sample with weak reactivity to ELIZA failed for viral recovery (Table 4-14), the titer of antibodies can reflect the degree of BoHV-2 infection, the titer of leukemic cattle are usually higher than aleukemic cattle, this agreed with Ferrer, *et al.,* (1974). The results of the current study also detected the difference between samples in their ability to grow in vitro. The virus from sample 162 was isolated directly after 3passage, consistently produced more viral antigen with tests, while the virus from sample 16 required 8 passages, such differences may related to the genetic or biological viral activity or differences in proviral load in cultured samples.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة القادسية

كلية الطب البيطري

دراسة سريرية وجزيئية عن مرض التهاب الحلمات التقرحي في الابقار في محافظة القادسية

اطروحة

تقدم بها إلى مجلس كلية الطب البيطري / جامعة القادسية جزءا ً من متطلبات نيل شهادة البكلوريوس في علم الطب والجراحة البيطرية

الباحثة

اسراء جواد كاظم

بإشراف

المدرس الدكتور
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١٤٣٩ هـ ٢٠١٨

2018م

الخلاصة

التهاب الحلم التقرحي البقري هو مرض مستوطن يصيب الماشية في العديد من البلدان، بما في ذلك العراق .وينجم عن فيروس ألفا الهربس الذي يسبب عدوى في جلد الماشية، الذي يتطور الى العديد من الأشكال المرضية المحتملة: نوع التقرحي الحادة ، التهاب الجلد السطحية ، وأمراض تكتل الجلد الكاذب .وذلك بسبب طبيعته القابلة للنقل ولها تأثير على صناعة الألبان . وركزت الدراسة على الجزأين، الجزء الأول شمل تحديد الجوانب الوبائية والسريرية، والجزء الثاني كان قد تلخص بشأن التشخيص الجزيئي ودراسة شجرة النشوء والتطور في محافظة القادسية في العراق، ولكن الفترة الضيقة من الدراسة وتأخر نتائج تسلسل منع بما في ذلك بعض من نتائج الجزء الثاني.

في الجزء الأول من الدراسة، بين الفحص السريري لحوالي ٢٠٠ من الأبقار المرضعة البالغة (١٠٠ من السلالة و ١٠٠ سلالة محلية) و ٥٠ عجول من محافظة القادسية، أظهرت النتائج سريريا أن ١٨ (٧.٢٪) من ٢٥٠ الماشية التي يم فحصها وجود الفات الحلم والضرع الآفات ومخطم الجول .وقد تم إصابة ثلاثة عشر (٦.٥٪) من الأبقار الحلوب، وكانت جميع الأبقار المصابة في فترة الرضاعة وخمسة (١٠٪) من العجول الذين تتراوح أعمارهم بين ١ أسبوع إلى ٣ أشهر اظهرول افات في المخطم والفم .الآفات التهاب الجلد السطحية الناجمة عن فيروس الهربس البقري في عجول تربية الألبان .سريريا، كانت جميع الماشية المتضررة في ٢-٦ سنوات من العمر، وكان التقرحات والقشور على العلامات السريرية التي لوحظت شملت قطعت

من الآفة السريرية الحادة، والتي تتميز بالتضخم وزيادة في حجم واحد أو أكثر من الحلمات، وعادة العدوى أثرت أربع حلمات ومددت الآفة إلى جلد الضرع .بشكل عام شدة الآفات تراوحت بين عدة قرحة مغطاة بإحكام تلتصق من الأصفر إلى اللون البرتقالي الجروح لقطع الآفات الحادة تشارك كلها الحلمات مع شكل غير منتظم من القرحة، الجرب والشقوق من الحلمات.

كشفت نتائج تفاعل البوليميراز سلسلة الجين بوهف-٢ في جميع العينات السريرية (٧.٧٥٪ .(كل ١٨ من العينات المصلية كانت ير إيجابية، والكشف الجزيئي يستهدف غب إنيس الذي أعطى الفرقة تتوافق مع ٦٠٧ bp القطاع.

في هذا المسح، اعتمادا على النتائج الجزيئية، كما تم التحقيق في بعض العناصر الوبائية مثل السن والسلالات ل بوهف-٢ .infectedولتحليل المخاطر المرتبطة بسلالة الماشية المصاحبة للعدوى بفيروس BoHV-2 - ٢، أصيبت سلالتان (سلالة متقاطعة وسلالة محلية) من الماشية العراقية .وقد تم إصابة كلتا السلالتين بنسبة ٨ (٨٪) و ٥ (٥٪) للسلالة المحلية والمتشابكة على التوالي .من الناحية الإحصائية أظهرت النتائج عدم وجود فروق ذات دلالة إحصائية في قابليتها للإصابة بوهف-٢ .infectionأظهرت نتائج هذه الدراسة أن عدد الحيوانات المصابة ينخفض مع تقدم العمر، وبلغ معدل الإصابة بين الأبقار ٢-٤ سنوات من العمر ١١٠/٩ (٢.٨٪)، في حين أن الأبقار أكثر من ٥ سنوات من العمر ٩٠/٤ .(4.4٪كما أظهرت النتائج وجود فروق ذات دلالة إحصائية بين أعمار الأبقار من حيث قابليتها للإصابة ب بونف-٢.

في الختام، فإن هذه الفيروسات المعزولة في هذه العزلات العراقية هي من أفراد عائلة بوهف-٢ و التي تشير إليها كلينكال، الجزيئية، هذه العزلات بوهف-٢ العراقية تمثل أداة لا تقدر بثمن كمصدر للدراسات المستقبلية وتوفير نظام استنساخ الخلايا استنساخه للدراسة ال التعريف، هربسفيروسيس