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Molecular and histopathological detection of lumpy skin disease in Buffaloes, Iraq

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Abstract---The study aim is to confirm infection of Iraqi buffaloes with the lumpy skin disease virus (LSDV) using of molecular conventional polymerase chain reaction (PCR) and histopathology. Totally, 150 buffaloes of different ages and sexes at three Iraqi provinces (Wasit, Dhi-Qar and Maysan) were subjected for collection of 5 ml blood samples and for clinical examination for detection of skin lesions and collection of tick samples during 2021 to March 2022. The total positive results for conventional PCR examination of 150 buffaloes was 8 (5.33%) using the blood samples. Among totally 13 suspected LSD skin lesions, only 1 (7.69%) sample was found to be positive. Among 29 tick samples collected from the 29 infested buffaloes, the findings revealed no positive samples in these samples. Histopathological analysis of nodular skin lesion showed that there were edema, hyperemia, acanthosis, severe hydropic degeneration, and hyperkeratosis in the epidermis; whereas in the dermis, there were mononuclear cell infiltration, inclusion bodies, and vasculitis. Conclusion: In Iraq, despite vaccination programs applied since the first outbreak in 2013, LSDV remains large in persistence and distribution among most areas of the country, resulting in apparent morbidities and mortalities. In our knowledge, the present study was the first one that dealt with LSD-related buffaloes. To support rare clinical signs, effective control of LSD requires an accurate and rapid laboratory diagnostic method like PCR and histopathology assay could be a potential method to identification and confirmation of the disease in concomitant with clinical examination.

Keywords---cattle, histopathology, Iraq, lumpy skin disease, polymerase chain reaction.

Introduction

Lumpy skin disease (LSD) is an acute to chronic bovine viral disease caused by LSD virus (LSDV) that classified under Capripox genus of *Poxviridae* family (Gharban et al., 2019). Initially, the disease that first reported in 1929 in Zambia is suspected to be resulted by either poisoning or hypersensitivity to an insect bite, until the infectious nature was recognized in 1943 (Koirala et al., 2022). Since then, LSD is spread to other southern and northern countries of Africa, and then Middle East including Iraq incurred enormous economic losses in cattle livestock (Alkhamis and VanderWaal, 2016). However, the causative pathogen transmits mainly by biting insects such as mosquitoes and biting flies which act as vectors and less commonly by direct contact with skin lesions, milk, nasal discharge, saliva, or semen of infected animals (Sprygin et al., 2019). LSD can vary from mild to severe form based on the virus's strain, host species and breed resulting in apparent clinical symptoms include fever, nasal and eye discharges, fall in milk yield, multiple nodules mainly under the skin of most body parts, and lymphadenopathy (Mulatu and Feyisa, 2018).

In endemic countries, economic losses are depending on the rates of morbidities and mortalities as there is no specific antiviral treatment available for infected animals. However, prevention of infection and protection of susceptible animals can be made by vaccination; whereas, supportive treatment for infected ones can effectively reduce the effects of disease (Babiuk, 2018; Zhugunissof et al., 2020). At the field, presumptive diagnosis depends mainly on the typical clinical signs and postmortem examination; whereas, at the laboratory, a combination of histopathological findings with the confirmatory techniques such as electron microscopy, serology, and molecular assays, and viral isolation can provide a definitive detection for LSDV (Abdallah et al., 2018; Gharban et al., 2019; Flannery et al., 2021). Polymerase chain reaction (PCR) is one of the most useful molecular tests which applied routinely to identify and confirm *Capripoxvirus* with high sensitivity and specificity (Sudhakar et al., 2020).

In Iraq, several outbreaks have been reported in bovine animals at many provinces throughout the past 10 years; however, all carried out studies were directed toward reporting and confirmation of LSD in cattle (Jarullah, 2015; Jameel, 2016; Mhemid, 2016; Mansour, 2017; Gharban et al., 2019; Alhakima et al., 2020). Hence, this study was designed to confirm of LSD infection in buffaloes using the molecular assay and histopathology for first time in Iraq.

Materials and methods

Ethical approval

The current study was approved by and carried out under the license of the Scientific Committee of the Department of Internal and Preventive Veterinary Medicine and the Scientific Committee of the College of Veterinary Medicine, University of Baghdad (Baghdad, Iraq).

Study animals

Totally, 150 unvaccinated LSD-buffaloes of different ages, both sexes and many regions were selected equally from three Iraqi provinces; Wasit, Dhi-Qar and Maysan. The selection of study animals was based on the information of veterinarians and owners about the recent appearance of clinical LSD cases in cattle and/or buffaloes existed at these areas. Initially, all study buffaloes were subjected to clinical examination to detect of pursued skin lesions and to collect of tick samples if available. All study animals were subjected to draining 5 ml of jugular venous blood into an anticoagulant EDTA tubes.

Molecular examination

Following the manufacturer instructions of the two DNA Extraction Kits, iNtRON Biotechnology (Korea) and Genaid (USA), DNAs were extracted from the samples of blood/skin lesions and ticks, respectively. The concentration (ng/ μ L) and purity of extracted DNAs were measured at an absorbance of 260 /280 nm using the Nanodrop system (Thermo-Scientific, UK). The mastermix tubes were prepared using the Maxime PCR Premix Kit (iNtRON Biotechnology, Korea) at a total volume of 20 μ L through targeting the *Envelop Protein 32 (P32)* gene [(F: 5'-AGGTTCGCGAAATTCAGATGT-3') and (R: 5'-TCCCCCTG TACGAATACA-3')]. The Thermal Cycler conditions were included 1 cycle initial denaturation (95°C / 5 minutes); 35 cycles of denaturation (95°C / 30 seconds), annealing (60°C / 30 seconds) and extension (72°C / 1 minute); and 1 cycle final extension (72°C / 5 minutes). The PCR products were analyzed by electrophoresis of the stained Agarose gel with Ethidium Bromide at 100 volt and 80Am for 1 hour. The positive samples were detected at an amplicon size of ~499bp.

Histological examination

For light microscopy, formalin fixed and paraffin-embedded procedure (FFPE) was used to prepare the tissue sections (Slaoui and Fiette, 2011; Al-Shaeli *et al.*, 2020). After 24 hours of tissue fixation, the 10% of NBF was discarded and consciously replaced each 12 hours interval. Then, NBF was replaced at 3rd day until subjection to dehydration step. To remove water from the tissue specimens, series of increasing concentrations of ethanol were used for dehydration that was carried out as following as 70% Ethanol for 15 minutes, 80% Ethanol for 15 minutes, 90% Ethanol for 15 minutes, and 100% Ethanol for 30 minutes. Xylene was used as a solvent agent to replace ethanol and to remove a substantial amount of fat from the tissue specimens. The clearing involved 3 steps by replacing tissues into xylene twice for 20 minutes, and then for 45 minutes. Different concentrations of liquid paraffin and xylene were used to infiltrate tissue specimens with paraffin wax as following as 75% xylene and 25% paraffin wax for 15 minutes, 50% xylene and 50% paraffin wax for 15 minutes, 25% xylene and 75% paraffin wax for 15 minutes, and 100% paraffin wax for 15 minutes at 60°C, and then allowed to be cooled at 20°C (repeated twice at 30 minutes and at 45 minutes). Tissue specimen of each sample was carefully oriented in plastic cassettes filled later with the molten paraffin wax at 60°C, and left to solidify at cold plate. Blocks of tissue specimens were sectioned by the microtome at a thickness of 4-5 micrometer (μ m). After the sectioning process, tissue sections

were floated in water bath at 45°C and mounted on a standard microscope glass slide. After mounting, the slides were removed directly from the water bath, allowed to vertically dry overnight at 37°C, and then stored in dry slide box at room temperature.

According to manufacturer instructions, tissue sections were stained as following as initially, Hemtaoxylin and Eosin working solutions were prepared and warmed at room temperature, and then filtered using filter paper. Differentiation solution was prepared by adding 1 ml of 37% HCL to 100 ml of 70% ethanol. Paraffin sections were dewaxed in xylene for 15-20 minutes, and rehydrated in ethanol alcohol (100%, 90%, and 70%) for 15 minutes at each concentration. The slides were washed in running tap water for 3 minutes and stained for 10 minutes in Hematoxylin. The slides were decolorized (differentiated) by adding one drop of acid alcohol for 2 seconds. The slides were washed carefully by the running tap water for 3 minutes, air dried, and stained with Eosin for 5 minutes, and then rinsed with tap water for 30 seconds. The slides were dehydrated with ascending concentrations of ethanol alcohol (70%, 90%, and 100%) at 2 minutes interval for each concentration. The slides were cleared in xylene two times for minutes each, and then, 1 drop of Canada-Balsam was added to each slide over the tissue sections, and covered with cover slip. After adding 1 drop of immersion oil, slides were examined using the light microscope, under an objective lens, at X10 and X40.

Statistical analysis

The findings of present study were analyzed using the GraphPad Prism version 6.0.1.298 (*GraphPad Software Inc., USA*) software. Chi-square (χ^2) was applied to detect significant differences between values of clinical signs of positive and negative animals by molecular assays. Odds ratio was applied to estimate statistical association between age and sex factors and positive results by both CR assays. Values were represented as mean $\pm$ standard errors (M $\pm$ SE) or percentage (%), and the statistical differences in study results were considered significant at a level of (P<0.05)

Results

The total positive results for conventional PCR examination of 150 buffaloes was 8 (5.33%) using the blood samples. Among totally 13 suspected LSD skin lesions, only 1 (7.69%) sample was found to be positive. Among 29 tick samples collected from the 29 infested buffaloes, the findings revealed no positive samples in these samples (Figures 1, 2).

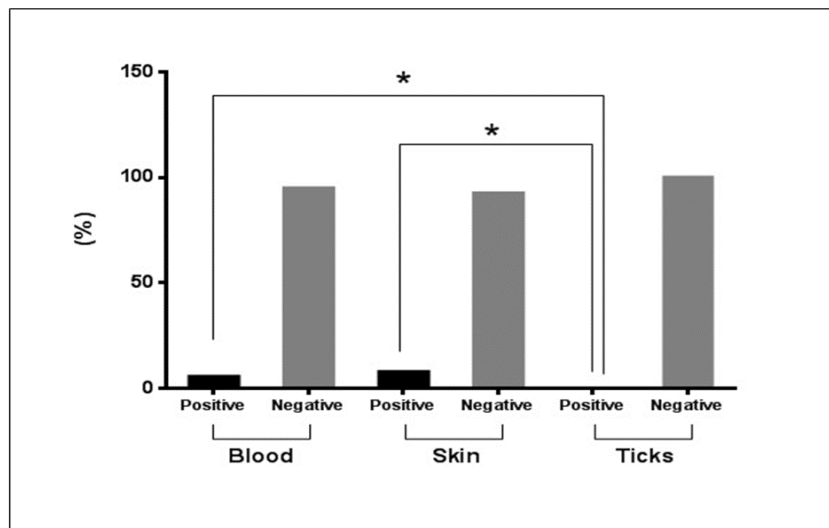


Figure (1): Total results for conventional PCR examination of 150 blood samples, 13 skin lesions and 29 tick samples using the qualitative conventional PCR assay. Significance * ($P < 0.05$)

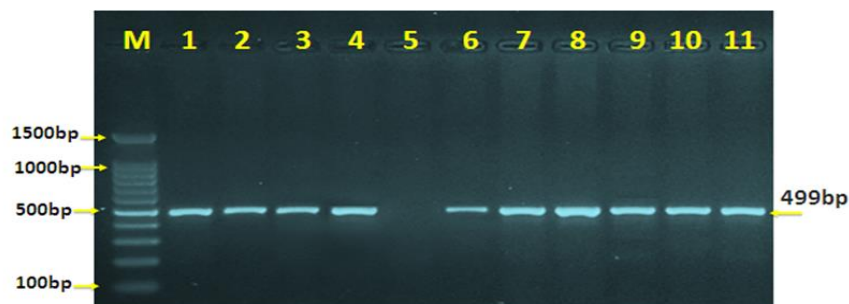
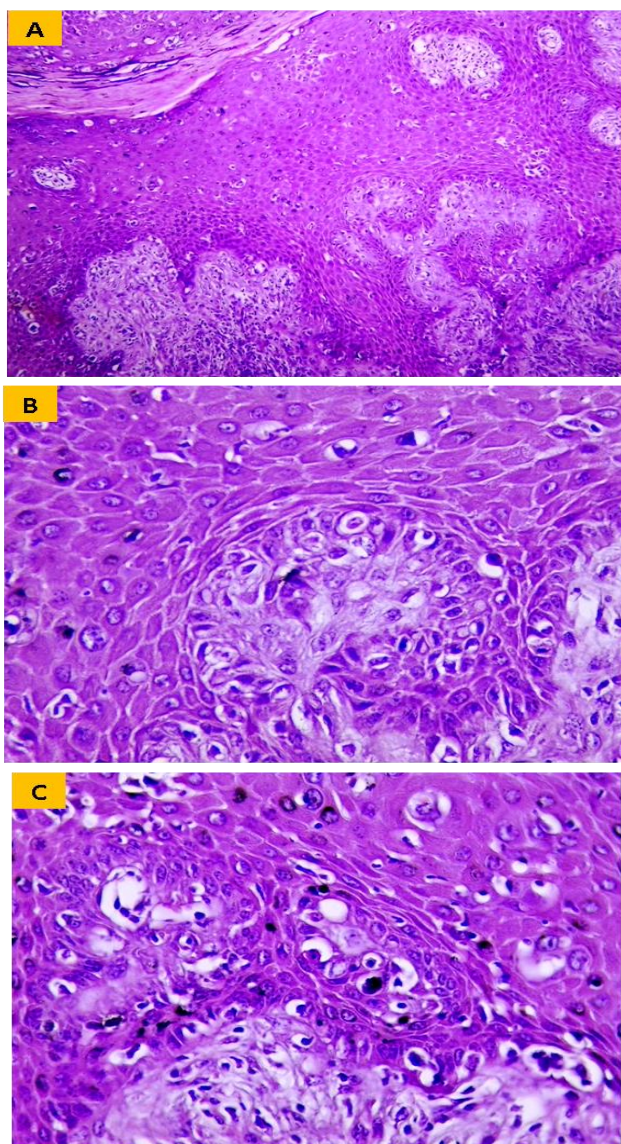


Figure (2): Electrophoresis of PCR products for envelope protein (P32) gene at 499bp using 1.5% Agarose gel stained with Ethidium Bromide at 100 Volt, 80 Am for 1 hour.

M: Ladder marker (100-1500bp); Lane (4): Positive Control; Lane (5): Negative Control; Lane (1): Positive skin lesion sample; Lanes (2, 3, 6, 7, 8, 9, 10 and 11): Positive blood samples

Histopathological analysis of nodular skin lesion showed that there were edema, hyperemia, acanthosis, severe hydropic degeneration, and hyperkeratosis in the epidermis; whereas in the dermis, there were mononuclear cell infiltration, inclusion bodies, and vasculitis (Figure 3).



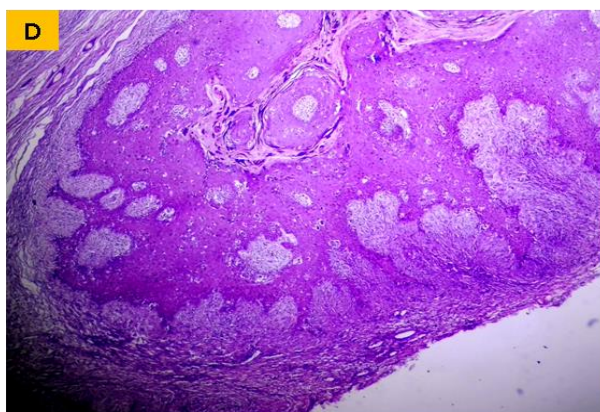


Figure (3): Histopathological examination of buffalo's positive skin lesion with LSD
The tissue stained with Hematoxylin and Eosin stain, examined under light microscope and images (A & D) were at X10; while, images (B & C) were at X40

Discussion

LSD is an exhausted viral disease affecting many countries, particularly at Africa and Asia resulting economically in great losses due to the high rates of morbidity, chronic debilitation in diseased cattle, abortion, severe reduction in milk production, weak growth, damage to hides, and temporary or permanent sterility (Gharban et al., 2019). In addition, it is considered one of the transboundary animal diseases for its significant impact on trade and food security and the ability for spreading to other countries (Abutarbush et al., 2015). The real danger of the disease lies in the fact that it has continued to spread and to extend in its range to include new areas, countries, and fields (Tageldin et al., 2014). In Iraq, many outbreaks have been reported during the last 10 years, which varied in their severity and incidence; nonetheless, it is unclear how the disease is maintained during interepidemic periods (Gharban et al., 2019). It thought that LSDV could preserve by either the unapparent infections cycled in cattle or old lesions or the role of wildlife animals in pathogenesis (Van Vuuren and Penzhorn, 2015). However, it is claimed that the very young calves, lactating cows, and animals suffering from malnutrition were developed generally the most severe infections probably due to an impairment of cellular immunity (Tageldin et al., 2014). Besides, the high ambient temperature coupled with farming practices to produce high milk might be deemed for stressing of these animals and contribute to the severity of the disease (Hunter and Wallace, 2001).

PCR examination for extracted DNA from skin lesions of study's cattle using PCR assay, Gharban et al. (2019) revealed that 90.14% and 60.56% were positives for primers targeted *P32* and *TK* genes, respectively. It reported that the *P32* gene is the better candidate gene for differentiating between vaccine strains from field isolates and for illustrating genetic variations between LSDVs (Mafirakureva et al., 2017; Yousefi et al., 2018). Besides, it indicated that the possibility of this gene for confirmation up to 100% of representative infected cattle among skin biopsies providing high sensitivity and specificity in detection of LSDV (El-Kholy et al., 2008). Furthermore, biopsies of the scabs or skin lesions are more suitable, distinguishable and represented sample for diagnosis of LSD as they substantial, progress to sit-fasts and contain abundant DNA of LSDV (El-Nahas et al., 2011).

Negative samples could be due to either of the complete absence of LSDV in a specimen or deficient level of virus that presents in tissue sample below the sensitivity of the assay (Gharban et al., 2019).

Although histopathological findings of LSDV varied in their characteristics considerably depending on the developmental stage, they can provide a basis for diagnosis (Al-Salihi, 2014; Mulatu and Feyisa, 2018). In the current study, histopathology of skin nodules showed that most cutaneous lesions were extended throughout all layers of skin and illustrated a profuse hyperkeratosis, acanthosis with marked vacuolation, cloudy swelling of granulocytes in stratum granulosum, and downward hyperplasia of stratum basale. Moreover, infiltration of inflammatory cells among the layers of dermis and thickening in walls of hair follicles was observed. Eosinophilic intracytoplasmic LSD viral inclusion bodies were detected microscopically within the cytoplasm of granulocytes in stratum basale, and some lesions appeared as papillary projections due to the proliferation of cells in stratum basale. In acute stages, LSD can comprise the granulomatous reactions in dermis and hypodermis, which extended to surrounding tissues, with the existence of vasculitis, lymphangitis, and thrombosis, and infarction that resulted in necrosis and edema. The presence of intracytoplasmic eosinophilic inclusions can be considered as a hallmark for acute and subacute stages of LSD lesions (Coetzer and Tuppurainen, 2004). As detected by Babiuk et al. (2008), it found that there was a significant variation in microvesicles, hyperkeratosis or acanthosis, presence of eosinophils, and the prominent vascular changes among skin lesions of naturally infected cattle. The variation could be due to the differences in disease progression stage at which the samples were collected or virus strain differences (Tageldin et al., 2014). In the field, numerous skin diseases can persist, which confusing the diagnosis of LSD as pseudo-LSD that could be differentiated clinically by lack of systemic signs and histologically as the lesions will involve only the epidermis and leave scabs after sloughing. Urticaria, tick bites, and insect bites/stings may confuse with LSD but can be differentiated by the absence of eosinophils and presence of deep vasculitis with intracytoplasmic inclusion bodies in keratinocytes and epithelium of hair follicles (Brenner et al., 2009; Gharban et al., 2019).

Conclusion

In Iraq, despite vaccination programs applied since the first outbreak in 2013, LSDV remains large in persistence and distribution among most areas of the country, resulting in apparent morbidities and mortalities. In our knowledge, the present study was the first one that dealt with LSD-related buffaloes. To support rare clinical signs, effective control of LSD requires an accurate and rapid laboratory diagnostic method like PCR assay that considered the best available test of choice for the identification of the disease.

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