

Molecular detection of lumpy skin disease virus in cattle by polymerase chain reaction in Iraq

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Summary

This study is conducted to detect lumpy skin disease virus in Babylon, Al-Qadysia and Al-Muthana governorate during autumn 2014 using conventional polymerase chain reaction. A total of 150 specimens: 50 whole blood samples, 50 skin nodular biopsies and 50 tick samples were collected from infected animals of different breeds, genders and ages during lumpy skin disease outbreak. The results revealed that 104 cases (69.33%) were positive for lumpy skin disease virus by using polymerase chain reaction, with significant ($P < 0.05$) differences between positive and negative cases. Out of 50 blood samples, 22 cases (44%) were positive for lumpy skin disease virus. The nodular skin samples collected from slaughtered animals showed 36 positive cases (72%), whereas 46 tick samples (92%) were positive for the disease, with significant ($P < 0.05$) difference among them. According to gender, the finding showed significant results of lumpy skin disease in females (78.78%). It was recorded that higher percentage of positive cases was found in Friesian cattle (100%), crossbreed (73.58%) while native breed was (50.76%) with significant ($P < 0.05$) difference among them. Regarding age groups, the results showed that all ages were susceptible to lumpy skin disease and significantly not different.

Keywords: Lumpy skin disease, Cattle, Polymerase chain reaction, Skin nodules.

Introduction

Lumpy skin disease (LSD) is a serious devastating significant transboundary, emerging skin viral disease of cattle (1) caused by lumpy skin disease virus (LSDV) which is termed as Neethling virus of the genus *capripoxvirus* within *Chordovirinae* of family *poxviridae* (2). Various strains of *capripoxvirus* are responsible for the infection which are antigenically and serologically closely related to the strains causing sheep and goat poxvirus but distinct at the genetic level (3 and 4). There are differences between geographic distribution of sheep poxvirus, goat poxvirus and lumpy skin disease, suggesting that cattle strains of capripoxviruses do not infect and transmit between sheep and goat (3 and 5). It is acute to subacute or inapparent disease with variable severity depending on strain and host breed (1 and 2).

Lumpy skin disease is included in the list of Notifiable Diseases of OIE and because of the direct and indirect product losses it is estimated to be as high as 45-60% (6). The disease have the ability to compromise food security through protein loss, reduced output of animal production, increased costs of disease control, reduced milk yield, weight

loss, abortion, mastitis and infertility in both sexes (7). Permanent damage to the skin and hide greatly affect leather industry. It causes ban on international trade of livestock (8). The disease could be suspected whenever clinical signs indicate high fever that might exceed 41°C, enlarged peripheral lymph nodes, excessive salivation, lacrimation and nasal discharge, disseminated appearance of pox lesions in the skin, mucous membrane and internal organs (9). Lumpy skin disease occurs across Africa and has recently been aggressively spreading in the Middle East including Iraq (8). It has now spread to almost all the regions and agro ecological zones, with a potential threat to become established in other parts of the world (10-12). The study showed molecular evidence of transstadial and transovarial of LSDV by hard (ixodid) tick *Rhipicephalus decoloratus* and mechanical or intrastadial transmission by *Rhipicephalus appendiculatus* and *Amblyomma hebraeum* (13 and 14).

Regarding diagnostic methods, several serological and molecular tests are used for the successful control and eradication of LSD. Serological assays are suitable for survey, but these assays are not sufficiently reliable or fast

to be used as primary test. Different molecular tests are currently replaced other less sensitive and slower diagnostic methods such as conventional PCR, real time PCR, and loop-mediated isothermal amplification assays (12). The aims of the study includes: Molecular detection of LSDV, DNA from clinically infected animals in skin specimens, whole blood samples and the biting ticks by using conventional PCR assay.

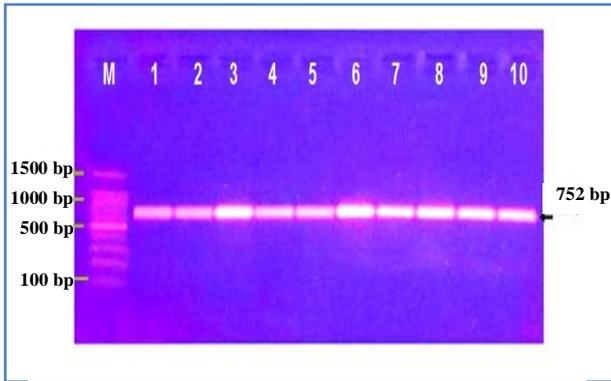
Materials and Methods

The study was carried out on 150 clinically infected cattle of both sexes with different ages (less than 1 year to 10 years) and breeds (Friesian, cross and local breeds) during the outbreak of the disease during the period of September, October and November 2014. Animal samples were collected from Babylon, AL-Qadysia and AL-Muthana governorates. Whole blood samples were collected from 50 clinically infected animals during the first week of symptoms. Five to ten milliliters of whole blood were collected from jugular vein, were placed in EDTA anticoagulant evacuated tubes Different size of ticks were collected randomly from animals showed nodular lesions and sitfast covering all the body. Fifty skin nodules were collected from infected animals brought to the slaughter house, some of these animals showed lacrimation, salivation, enlargement of superficial lymph nodes and the nodular eruption measured 2-5cm in diameter and others showed sitfast lesions. Samples were transported to laboratory in cool bag, then kept under -20 °C for DNA extraction Genomic DNA was extracted from blood, ticks and skin nodular tissues of infected cattle by using AccuPrep® Genomic DNA extraction, kit (Bioneer. Korea), and was done according to company instructions. The PCR assay was performed to amplify the P32 gene that used in detection of lumpy skin disease virus from infected skin lesions, ticks, and blood of cattle. This assay was carried out according to (15). The PCR primers were designed by using the complete sequence of Lumpy skin disease virus P32 antigen gene (GenBank: AF124516.1). The NCBI Gene-Bank data base and Primer 3 plus online, used and supplied by (Bioneer Company, Korea). Conventional

PCR was carried out with forward P32 primer 5'-CGCGAAATTCAGATGTAGTTCCA-3' and Reverse primer 5'- TGAGCCATCCAT TTTCCA ACTC-3'. The PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250 µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, MgCl₂ 1.5 mM, stabilizer, and tracking dye). The PCR master mix was prepared according to kit instructions. A total volume of 20 µl have done by adding 5 µl of purified genomic DNA and 1.5 µl of 10 pmole of forward primer and 1.5 µl of 10 pmole of reverse primer, then complete the PCR premix tube by PCR water into 20 µl then mixed by Exispin vortex and centrifuged for 3000 rpm for 3 minutes. The reaction was performed in a programmable thermocycler as follows: one cycle of initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 30 cycles of annealing at 58 °C for 30 s, 30 cycles of extension at 72 °C for 1 min. and then one cycle of final extension at 72 °C for 10 min. The PCR products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator The data of present study were analyzed by SPSS program (version 20) software 2011, using Chi- square test and P values of (P≤0.05) were considered as statistical significant (16).

Results and Discussion

The viral DNA was successfully extracted from 150 DNA extracted samples and was utilized for conventional PCR. The amplicon size of PCR product in positive samples had a molecular weight of 752 bp (Fig. 1). A hundred and four cases were positive for the LSD infection 69.33% (Table, 1). Different samples of nodular lesions, blood and tick samples were tested by the PCR. The viral DNA was detected in 36 samples (72%) out of 50 skin nodular lesions, 22 (44%) out of 50 whole blood samples collected from infected cattle and in 46 (92%) from 50 tick samples collected from clinically infected cattle. Significant (P<0.05) differences between positive and negative cases was recognized, beside that there was statistical differences among the various samples.



Figure, 1: Agarose gel electrophoresis image that show the PCR product analysis of P32 gene in lumpy skin disease. Where M: marker (1500-100 bp), lane (1-4) positive skin lesion samples, lane (5-7) positive blood samples, and lane (8-10) positive ticks samples, at 752 bp PCR product.

Table, 1: Detection of LSDV DNA by conventional PCR in cattle.

Samples	Number of tested samples	Positive DNA detection	Percent (%)	Negative DNA detection	Percent (%)
Skin	50	36	72 ^a	14	28 ^a
Blood	50	22	44 ^b	28	56 ^b
Ticks	50	46	92 ^c	4	8 ^c
Total	150	104	69.33 ^A	46	30.66 ^B

Chi-square at significance level (^{a'b'c} $P \leq 0.05$) Different capital letters represent significant difference ($P < 0.05$) horizontally while different small letters represent significant difference vertically.

Table (1) the percentage of total positive cases was high because of the occurrence of the disease for the first time in Iraq, suggesting the presence of disease as an outbreak, which was in agreement with the findings (17). Moreover, (18) pointed that the high percent of positive cases could occur under certain circumstances. The PCR technique detected LSDV in 36/50 (72%) skin biopsies from clinically infected cattle. The result of the present investigation was lower than the finding recorded by (19 and 20) in which the percentage of positive skin biopsies was 100%. The 28% failure in skin lesions is probably due to the collection of some nodules in the convalescent stage or due to presence of other diseases that clinically confused with LSD such as pseudo-lumpy skin disease and bovine herpes virus-2.

The detection rate of LSDV in EDTA blood were 44% in samples collected during febrile stage. The result was relatively higher than the finding of (20) in which the detection rate in blood was 40%. The 56% failure in detection of the virus in viremic animals is probably due to the fact that LSDV is present

at low infective titers in blood during the first days following infection. Because of such low LSDV titers in blood, vectors are more likely to mechanically transmit the LSDV following contamination of their mouthparts with the virus from skin lesions (21 and 22). Lumpy skin disease viral DNA were detected in a high percent 92% in tick samples followed by the samples taken from skin biopsies 72%. The presence of LSDV nucleic acid in tick samples in a very high level might assure the incrimination of the vector in the epidemiology of LSD. This is supported by (23), who proved that LSD viral DNA is present in a high level, might reach 100% in field ticks collected from naturally-infected cattle, which might play a role in maintenance of the virus in ticks and environment.

According to the gender, the result showed that the higher percentage of lumpy skin infection reported in females 78.78 %, whereas the percentage in males was 50.98 % (Table, 2). Statistically, there are significant differences between them ($P \leq 0.05$). Present results found to be in agreement with the finding recorded by Salib and Osman (17 and 24), who reported that LSD viral infection was significantly more frequently observed in females than in male cattle. This might be attributable to stress factors of exhaustion and fatigue in addition to the physiological reasons such as lactation and pregnancy. Gari (25), who pointed that the susceptibility of the disease was more obvious in males than females and as the authors explained due to stress factors using draft oxen power for the production of agricultural crops, animal manure, for fertilizer, milk and milk products for household consumption and sale.

Table, 2: Percentage of positive LSD samples according to gender by PCR in male cattle.

Sex	No. samples	Positive samples	Percent (%)
Male	51	26	50.98 ^a
Female	99	78	78.78 ^b
Total	150	104	69.33

(^{a'b} $P \leq 0.05$) Different letters represent significant difference ($P < 0.05$)

Diagnosis by using conventional PCR assay for detection of LSD samples of affected cattle in different breeds showed that the higher

percentage of positive cases was in Friesian cattle (100 %), then crossbreed 73.58 % while native (local) breed (50.76 %). The positivity rate in Friesian/crossbreed cattle was found to be significantly higher than in local breed (Table, 3). The finding of the present study was in agreement with (17, 24 and 25), who documented that Friesian and crossbreeds had statistically higher odds of becoming affecting than the local breed, and this might be due to breed susceptibility (26). Beside that Friesian and crossbreed showed more severe skin lesions compared to the local breed. Hunter and Wallace (27) claimed that animals suffering from malnutrition generally developed the most severe infections, probably due to impaired cellular immunity. As present in (Table, 3) local breed was less susceptible on comparing with Friesian and crossbreed, but statistically had a significant result (50.76 %) due to the recent occurrence of the disease in Iraq and no reports documented that before 2013. Generally, the LSD affect all cattle breeds because the virus showed host specificity as is supported by (28) found that the DNA genome contains 156 putative genes, from which nine genes might play a role in its ability to infect cattle breeds that is mean these genes are responsible for viral host and virulence.

Table, 3: Percentage of positive LSD samples according to cattle breeds by PCR.

Breed	No. samples	Positive samples	Percent (%)
Friesian	32	32	100 ^a
Crossbreed	53	39	73.58 ^b
Native (local)	65	33	50.76 ^c
Total	150	104	69.33

(^a^b^c P≤0.05). Different letters represent significant difference (P<0.05) .

Table (4) the percentage of positive cases of LSD between age groups of < 2years, 2-4 years and >4 years was 69.11%, 69.23%, 69.64%, respectively were not different with a probability of > 0.05. The finding of the this study was coincided with the results of (25 and 29) who proved that all age groups were invariably susceptible to LSD infection, but the result was inconsistent with the findings of (17 and 24), who reported that high morbidity rate and severity of the disease occur mainly in

young age category, because of the immune system is still primitive as compared with adult cattle

Table, 4: Percentage of positive LSD samples according to cattle ages by PCR.

Ages	No. samples	Positive samples	Percent (%)
< 2 years	68	47	69.11
2-4 years	26	18	69.23
> 4 years	56	39	69.64
Total	150	104	69.33

(P≤0.05) Similar letters represent non-significant differences (P < 0.05).

In conclusion, this study illustrated that the conventional PCR is simple and, rapid diagnostic method. Tick samples and skin biopsies were favourable for detection of LSD viral DNA. Local breed was less susceptible than the Frisian and cross breed. Females were more sensitive than males, whereas all age groups were infected with severe and serious complications of the disease.

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الكشف الجزيئي لفايروس مرض العقد الجلدي الحديث في الأبقار باستعمال تقنية تفاعل انزيم البلمرة المتسلسل في العراق

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الخلاصة

أجريت هذه الدراسة لكشف فايروس مرض العقد الجلدي في محافظات بابل والقادسية والمثنى في فصل الخريف للعام 2014 باستعمال تقنية تفاعل انزيم البلمرة المتسلسل. جمعت 150 عينة من أبقار مصابة من مختلف السلالات والأعمار ومن كلا الجنسين منها 50 نموذج دم و50 خزعه جلدية و50 قرادة وذلك خلال تفشي مرض العقد الجلدي. أظهرت نتائج التشخيص باستعمال تقنية تفاعل انزيم البلمرة المتسلسل بوجود 104 حالة (69.33%) موجبة لمرض العقد الجلدي مع ظهور اختلافات معنوية (احتمال > 0.05) بين الحالات الموجبة والسالبة. من 50 نموذج دم ظهرت 22 حالة (44%) موجبة لفايروس مرض العقد الجلدي. أظهرت عينات العقد الجلدية التي جمعت من حيوانات مذبوحة أن 36 (72%) منها كانت موجبة، فيما كانت 46 (92%) عينة قراد موجبة للفايروس مع وجود اختلاف معنوي (احتمال > 0.05) فيما بينها. وفيما يتعلق بالجنس ظهرت نتائج معنوية لمرض العقد الجلدي في الإناث (78.78%). كما سجلت نسب عالية المعنوية لحالات المرض في سلالة أبقار الفريزيان (100%) تلتها الأبقار الهجينة (73.58%) فيما بلغت النسبة (50.76%) في السلالة المحلية وكانت الفروق بينها معنوية احصائياً. فيما يتعلق بالمجموعات العمرية أظهرت النتائج أن جميع الأعمار كانت قابله للإصابة بمرض العقد الجلدي والاختلافات بينها غير معنوية.

الكلمات المفتاحية: مرض العقد الجلدي، الأبقار، تقنية تفاعل انزيم البلمرة المتسلسل، العقد الجلدية.