Isolation and identification of lumpy skin disease virus from cattle on chorioallantoic membranes (CAMs) of fertile eggs

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Abstract

Lumpy skin disease (LSD) is an important infectious viral skin disease of cattle causing high economic losses. In the present study, isolation of lumpy skin disease virus from 67 samples was carried out via chorioallantoic membranes (CAMs) of embryonated chicken eggs (ECEs) aged 9 days. Five egg passages were carried out for each sample. These samples include 25 skin lesions, 12 nasal swabs, 12 heparinized blood and 18 internal organs samples. The samples were collected from clinically diseased and slaughtered cattle showed clinical signs believed to be LSD. Hyperimmune serum was prepared against reference LSDV (Kenyan strain). The isolated virus was identified using the prepared hyperimmune serum by using agar gel precipitation test (AGPT), latex agglutination test (LAT), reverse Passive haemagglutination test (RPHA). The positive results by AGPT, RPHA and LAT for field samples were 20 (30%), 35 (52%) and 40 (60%), respectively while after 5th passage in ECEs were 24 (53.8%), 39 (58%) and 43 (64%), respectively. For confirming isolated virus identification, six isolates were selected to perform neutralization test in ECEs. The six selected isolates for neutralization test were positive. The results indicate sensitivity of RPHA and LAT over AGPT in virus detection and neutralization test could be used for LSDV identification in ECEs. The isolated LSDV have no ability for haemagglutination of 1% bovine, sheep, chicken and rabbits RBCs.

Keywords: Lumpy skin disease virus, isolation, chorioallantoic membrane, latex agglutination, reverse passive haemagglutination.

Introduction

The Capripoxvirus genus is comprised of lumpy skin disease virus (LSDV), sheep poxvirus (ShPV) and goat poxvirus (GPV), causing disease in cattle, sheep or goat, respectively **(Esposito and Fenner, 2001).** Lumpy skin disease is an acute, subacute or inapparent viral disease of cattle and occasionally buffaloes characterized by pyrexia, generalized skin and internal pox lesions, and generalized lymphadenopathy **(Prozesky and Barnard, 1982, Davies, 1991 and Hamoda et al., 2002).** The disease is endemic in Central and South Africa. The first report of LSD outside Africa was from Kuwait in 1986-1988 **(Anonymous, 1988),** followed by Israel in 1989 **(Shimshony, 1990).** In Egypt, the LSD was first appeared in Suez Governorate after cattle importation from Somalia followed by Ismailyia Governorate in 1988 **(House et al., 1990)** and two disease outbreaks were reported in 2005 and 2006 **(Younis and Aboul Soud, 2005 and OIE, 2006)**. The disease was considered a "list A" disease by the Office International des Epizooties (OIE) due to its potential for rapid spread and ability to cause sever economic losses. The disease causes significant economic loss due to hide damage, loss of milk production, mastitis, infertility and death **(Weiss, 1968).** The disease is primarily transmitted by biting insects, particularly blood feeding insects, such as the mosquito. Contact transmission between animals may occur at low rate but can not be considered to play a significant role in transmission during epizootics (**Coetzer et al., 1994 and Chihota et al., 2001).**

Diagnosis of the disease is depend initially on clinical signs and definitive diagnosis is provided by virus isolation or its demonstration by electron microscope and identification of antigen by fluorescent antibody, serum neutralization, agar gel precipitation, antigen capture ELISA, Dot ELISA and immunoperoxidase (**Wood, 1988, El-Bagoury et al., 1995, Tuppurainen, 2005 and Younis and Aboul-Soud, 2005**). Polymerase chain reaction (PCR) assay has been described for detection of LSDV (**Ireland and Binepal, 1998, Hein et al., 1999, Tuppurainen, 2005 and Ibrahim, 2006**). Following diagnosis of the disease, rapid performing of control measures such as slaughter, ring vaccination and movement restrictions are required to limit losses (**Carn, 1993**).

The present study aimed to isolate lumpy skin disease virus from cattle in Egypt using embryonated chicken eggs, identification of the isolated virus using agar gel precipitation test, latex agglutination test, reverse passive haemagglutination and neutralization test and studying the haemagglutinating ability of the isolated virus.

Materials and Methods

Collection of samples:

Twenty-five skin lesions samples including skin nodules and scabs, 12 nasal swabs, 12 blood samples and Portions of lymph nodes (9) (prescapular, prefemoral and bronchial lymph nodes), lungs (4), kidneys (3) and livers (2) were collected from clinically diseased and slaughtered cattle in Dakahlia Governorate showed clinical signs believed to be LSD in form of multiple skin nodules or scabs either localized or generalized on the whole of the animals body with or without systemic signs (Figure 1,2and 3) for LSDV isolation.

Preparation of the collected field samples for virus isolation:

Skin samples and different internal organs:

They were prepared according to the Diagnostic Tests and Vaccines for Terrestrial Animals **(*OIE, 2004*).**

Anticoagulated (heparinized) blood samples:

They were prepared according to **Carn and Kitching, (1995b).**

Nasal swabs:

They were prepared according to **Mahy and Kangro, (1996)**.

Inoculation of the prepared samples on chorioallantoic membrane(CAM) of embryonated chicken eggs(ECEs):

It was carried out according to **Van Rooyen et al., (1969).**

Titration of isolated virus in ECEs (according to Van Rooyen et al., (1969):

For virus titration, twelve virus isolates from skin (3), internal organs (4), nasal swabs (3) and heparinized blood (2) samples that gave clear pock lesions on CAMs of ECEs were selected. The virus was titrated according to formula of **Reed and Muench, 1938.**The titre was expressed as $\log_{10}$ EID_{50}/0.1ml.

Preparation of hyperimmune serum against standard reference LSD virus:

It was done according to **Davies, (1982)** as follows:

Five rabbits were inoculated weekly with 0.5 ml of reference LSD virus having a titre of 10^5 $TCID_{50}$ per ml emulsified with equal volume of Freunds incomplete adjuvant subcutaneous, repeated 5 times. Then a final intravenous inoculation of 1 ml of LSD virus was given as shown in table (1) and the rabbit bled out ten days later and the hyperimmune serum was separated by centrifugation at 3000rpm for 10 minutes and kept at -20°C till used.

Table (1): The rabbit's immunization scheme for hyperimmune serum production against LSDV:

Injection No.	Route of injection.	Dosage of injection (ml).
1	Subcutaneous (S/C)	0.5 ml(with 0.5 ml adjuvant)
2	Subcutaneous (S/C)	0.5 ml(with 0.5 ml adjuvant)
3	Subcutaneous (S/C)	0.5 ml(with 0.5 ml adjuvant)
4	Subcutaneous (S/C)	0.5 ml(with 0.5 ml adjuvant)
5	Subcutaneous (S/C)	0.5 ml(with 0.5 ml adjuvant)
6	Intravenous (I/V)	1 ml (without adjuvant)

Identification of the isolated virus:

Agar gel precipitation test (AGPT):

It was carried out according to **Kitching et al., (1986)**.

Latex agglutination test (LAT):

It was carried out using Polybead® Amino 1.0 micron Microspheres (Polyscience, Inc) and Glutaraldehyde Kit (Polysciences,Inc).

a) Coupling of specific antibody to latex microspheres:

Latex particles were coated with antibody according to the manufacturer's instructions.

b) LAT procedure: It was done according to **Storch et al., (1988).**

Reverse passive haemagglutination (RPHA) test:

The isolated virus was identified using the prepared hyperimmune serum by RPHA according to **Scott et al., (1986) and Nachimuthu et al., (1995).**

Neutralization test (Alpha method, constant serum varying virus method):

Six isolates were selected to perform neutralization test. The test was carried out using ECEs (9 days old) according to **Koprowski and Lennette, (1946) and Beard, (1989).**

Haemagglutination ability of isolated LSDV:

The Haemagglutination test was carried out in microplates (V-shape) for determining the haemagglutinating ability of isolated Lumpy skin disease virus using 1%washed bovine, chicken, rabbits and sheep RBCs. It was done according to **Annon, (1971).**

RESULTS

Results of isolation of the virus on CAMs of ECEs

Number of samples that gave positive result in form of macroscopic lesions in form of thickening, congestion and swelling with small, opaque white lesions at site of inoculation or gray and opaque pock lesions that arranged in streaks and pin point in size and their percentage were recorded in **table (1) and figure (4 and 5).**

Table (1): Results of isolation of the virus from skin, nasal swabs, heparinized blood and internal organs on CAMs of ECEs:

Sample type		No. of collected specimens	1st passage positive specimens		2nd passage positive specimens		3rd passage positive specimens		4th passage positive specimens		5th passage positive specimens	
			No.	%	No.	%	No.	%	No.	%	No.	%
Skin		25	5	20	5	20	9	36	15	60	16	64
Nasal swabs		12	2	17	2	17	4	33	5	42	5	42
Internal organs	Lymph node	9	2	22	2	22	4	44	6	67	6	67
	Lung	4	0	0	0	0	1	25	3	75	3	75
	Kidney	3	1	33	1	33	1	33	1	33	1	33
	Liver	2	0	0	0	0	1	50	2	100	2	100
	Total	18	3	17	3	17	7	39	12	67	12	67
Heparinized blood		12	0	0	1	8	3	25	4	33.3	4	33.3
Total		67	10	15	11	16.4	23	34.3	36	53.7	37	55.2

Concerning the effect of the virus on embryos of inoculated ECEs , there was no effect on embryos after 1st , 2nd , 3rd or 4th passages but after 5th passage there were numbers of inoculated embryos showed death within 5 to 7 days post inoculation, multiple hemorrhages, oedema, stunted growth and abnormal feathering (figure 6) as observed in skin samples number 4, 6, 9, 14, 18, 19and 20, nasal swabs number 4, 5 and 9, heparinized blood sample number 9, Lymph node samples number 1, 3 and 9 and lung samples number 2 and 3.

Titration of isolated virus on CAMs of ECEs:

Results of isolated virus titration recorded in **table (2).**

Table (2): Titration of virus isolated from skin, internal organs, nasal swabs and heparinized blood samples after 5th passages on CAMs of ECEs:

Type of samples		Sample no.	Titre(log_{10} EID_{50}/0.1 ml)
Skin isolates		1	4.8
		6	3.2
		9	6
Internal organs isolates	Lymph node	2	5.3
	Lung	4	4.1
	Liver	1	5
	Kidney	2	3.2
Nasal swabs isolates		1	4.5
		5	3.2
		9	4.8
Heparinized blood isolates		5	3
		9	2.2

From results recorded in table (2), it was clear that the isolated virus from skin lesion has a maximum titre (10^6 EID_{50}/0.1 ml) followed by isolated virus from lymph node ($10^{5.3}$ EID_{50}/0.1 ml), isolated virus from nasal swabs samples ($10^{4.8}$ EID_{50}/0.1 ml) and isolated virus from heparinized blood has the lower titer (10^3 EID_{50}/0.1 ml).

Identification of isolated virus by AGPT, LAT and RPHA:

Collective results of identification of the virus in the original field samples and after 1st and 5th egg passages by AGPT, LAT and RPHA are showed in table (3) and figure (7 and 8):

Table (3): Comparative results of the virus identification in prepared field samples, after 1st passage and after 5th passage using AGPT, LAT, and RPHA test:

Type of sample	No. of samples	Number positive (percent positive) in field samples			Number positive (percent positive) after 1st passage			Number positive (percent positive) after 5th passage		
		AGPT	LAT	RPHA	AGPT	LAT	RPHA	AGPT	LAT	RPHA
Skin	25	9 (36)	14(56	16(64)	2(8)	7(28)	9(36	8 (32)	16(64)	18(72)
Nasal swabs	12	2(16.7)	4(33)	5(42)	0(0)	2(17)	3(25)	4(33)	7(58)	8(66.7)
Internal organs	18	9 (50)	13(72)	13(72)	1(5.6)	3(17)	5(28)	9(50)	12(67)	12(67)
Heparinized blood	12	0 (0)	4(33)	4(33)	0(0)	1(8)	2(17)	3(25)	4(33)	5(42)
Total	67	20(30)	35(52)	40(60)	3(4.5)	13(19.4)	19(28.4)	24(35.8)	39(58)	43(64)

Neutralization test (alpha method):

The neutralization index (NI) was expressed as the difference between the virus titre and its titre after addition of positive hyperimmune serum. Results of neutralization test were recorded in table (4).

Results obtained in table (4) showed that all tested samples were positive, NI ($\geq$ 1.5) while negative results were recorded in control sample which had no appreciable index.

Table (4): Neutralization indices (NI) of six selected virus isolates:

Type of samples	Sample no.	No.of inoculated eggs	Number of inoculated eggs showed pock lesions on CAM with serum plus isolated virus dilution							Titre of virus serum mixture (log_{10} EID_{50}/0.1 ml)	Titre of virus alone (log_{10} EID_{50}/0.1 ml)	NI*
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}			
Skin isolates	1	5	3	3	2	0	0	0	0	2.2	4.8	2.6
	9	5	5	3	3	1	0	0	0	3	6	3
Lymph node isolate	2	5	5	3	2	0	0	0	0	2.7	5.3	2.6
Lung isolate	4	5	3	3	1	0	0	0	0	2	4.1	2.1
Nasal swab isolate	9	5	4	4	2	1	0	0	0	2.8	4.8	2
Heparinized blood isolate	5	5	2	2	1	0	0	0	0	1.4	3	1.6

*** NI (Neutralizing index)** $\geq$ 1.5 is considered as a positive result (**Cottral, 1978 and House et al., 1990**).

Haemagglutination test of isolated virus:

1% washed bovine, sheep, chicken and rabbits RBCs were used in haemagglutination test for determination of the haemagglutinating ability of the isolated virus. It was found that LSDV did not agglutinate bovine, sheep, chicken and rabbits RBCs.

Discussion

In this study, a trial for isolation of LSDV from collected samples from clinically diseased and slaughtered cows showed clinical signs believed to be LSD using ECEs(9-days old). Sixty-seven samples (25 skin lesions, 12 nasal swabs, 12 heparinized blood and 18 internal organs samples) were collected and prepared then subjected for virus isolation via CAMs of ECEs, five egg passages were carried out. Five skin samples, 2 nasal swabs and 3 internal organs samples gave macroscopic lesion on CAMs of ECEs in first passage, 5 skin samples, 2 nasal swabs, 1 heparinized blood sample and 3 internal organs gave macroscopic lesion on CAMs of ECEs in second passage, 9 skin samples, 4 nasal swabs, 3 heparinized blood samples and 7 internal organs samples gave macroscopic lesions after third passage, 15 skin samples, 5 nasal swabs, 4 heparinized blood samples and 12 internal organs samples gave macroscopic lesions after fourth passage and 16 skin samples, 5 nasal swabs, 4 heparinized blood samples and 12 internal organs samples gave CPE in fifth passage. These results were sustained by **Woods, (1947), Van den Ende et al., (1949), Alexander et al., (1957), El-Kanawaty, (1989), Ismael, (2000), Hamoda et al., (2002) and Ahmed et al., (2005)** who observed pock lesions on CAMs of inoculated ECEs and the lesion of the virus was maintained by serial passages. On the other hand **Van Rooyen et al., (1959) and Hassan, (1993)** failed to detect macroscopic lesions on CAMs of inoculated ECEs.

Concerning the effect of the virus on embryo of inoculated ECE, there were numbers of embryos after 5^{th} passages showing death within 5 to 7 days post inoculation, multiple hemorrhages, oedema, stunted growth and abnormal feathering. Our results are in partial agreement with **Van den Ende et al., (1949)** who reported that LSDV have an effect on chick embryo in form of shrunken featherless embryo tightly wrapped in its amnion and almost complete disappearance of amniotic fluid but disagreed with **Buxton and Fraser, (1977**) who mentioned that LSDV cause no chick embryo death.

Infectivity titration of eleven selected isolates that gave clear pock lesions was done on CAMs of ECEs according to **Reed and Muench,(1938**) which showed isolated virus from skin lesions has higher titre (10^6 EID_{50}/0.1ml) followed by isolated virus from lymph node samples ($10^{5.3}$ EID_{50}/0.1ml), isolated virus from nasal swabs ($10^{4.8}$ EID_{50}/0.1ml) and isolated virus from heparinized blood (10^3 EID_{50}/0.1ml). These results are inconcurrence with **Ismael, (2000), Hamoda et al., (2002) and Younis and Aboul Soud, (2005)** who titrated isolated LSDV on CAMs of ECEs. The highest titre of isolated virus obtained from skin lesions reveal that the higher propagation of virus take place in skin as described by **Bowden et al., (2008)** who mentioned that greatest replication of capripoxvirus occur in skin. The low titre of virus obtained from

heparinized blood is coincided with that recorded by **Carn and Kitching, (1995b)** that mentioned that the low titre of LSDV present in blood of animals during the pyrexial stage is not sufficient for mechanical transmission by biting flies feeding on blood alone and they must feed on skin lesions to obtain sufficient amount of virus for transmission because acute skin lesions contain high titre of virus that are sufficient to contaminate the mouth parts of biting insects. The high virus titre obtained from lymph node sample suggests that it might also be target site for capripoxvirus replication as described by **Bowden et al., (2008)**.

Isolation and titration of LSDV from nasal swabs explain the virus shedding in nasal secretion. Conceivably, such secretion could contribute to virus transmission by aerosol. This result is inagreement with **Kitching and Taylor, (1985**) who demonstrated the transmission of sheep poxvirus using an aerosol suspension and with **Coetzer et al., (1994) and Chihota et al., (2001)** who reported that contact transmission of LSD between animals may occur at low rate but can not be considered to play a significant role in transmission during epizootics but disagreed with **Davies, (1991)** who reviewed that contact transmission do not readily occur.

Detection of LSDV in original field samples took place by Agar gel precipitation test (AGPT), Reverse Passive haemagglutination test (RPHA) and latex agglutination test (LAT) was carried out and the obtained results revealed that by using AGPT, there are 9 skin lesions, 2 nasal swabs and 9 internal organs samples gave positive results. This result is in accordance with **Kitching et al., (1986), Salim, (1991), Ali, (1993) and El-Bagoury et al., (1995)** who showed that the soluble LSDV antigens gave precipitation reactions in AGPT and in contrast to **Munz and Owen, (1966)** who reported that it had not yet possible to demonstrate agar gel precipitation with the Neethling type virus. Using of RPHA revealed that there are 16 skin lesions, 5 nasal swabs, 4 heparinized blood and 15 internal organs samples gave positive results, while by using LAT, 14 skin lesions, 4 nasal swabs, 4 heparinized blood and 13 internal organs samples gave positive results. LAT is sensitive than AGPT and slightly less sensitive than RPHA, this result is in agreement with **Carpenter, (1965) and Meurman and Granberg,(1993)** who stated that sensitivity of agglutination tests (RPHA and LAT) over precipitation test (AGPT) in virus detection may be due to the RPHA being able to detect as little as 0.005 µg antigen per ml where as the precipitation test able to detect not less than 5µg per ml and LAT is sensitive and efficient for the detection of various antigen-antibody systems.

The RPHA test is simple, economical and effective as well as the LAT since a large number of samples can be tested using Known hyperimmune serum in a short period. RPHA takes a longer time than the LAT, but is much faster than the AGPT. So LAT and RPHA are practical tests for LSDV detection owing to their simplicity, ease of operation and rapid antigen detection **(Rao and Negi, 1997).**

Identification of isolated LSDV in CAMS of inoculated ECES was carried out by AGPT, RPHA and LAT. The obtained results revealed that after first

passage, there are 2 skin lesions and 1 internal organs samples gave positive results by AGPT, 9 skin and 5 internal organs samples gave positive results by RPHA and 7 skin and 3 internal organs samples gave positive results by LAT. Number of positive samples increased after the 5th passage to be nearly equal or less than the number in field original samples. The results in case of heparinized blood samples and nasal swabs revealed that number of positive samples after the 5th passage more than those in case of field original samples. So detection of LSDV in skin and internal organs field samples is superior to detection after the five passages but in case of heparinized blood samples and nasal swabs, detection of the virus after the five passages is superior to direct detection on field samples. This may be due to high concentration of virus in skin lesions and internal organs and poor replication of virus in CAM after first passage and increase gradually till 5th passage but not reach to the same concentration in the field samples but low concentration of virus in nasal swabs and heparinized blood samples and after the 5th passage the concentration of virus become more than that in field samples. This result is in agreement with **Salim, (1991)** who stated that application of AGPT on inoculated CAMS after 5th passage was not superior to direct test on skin biopsies obtained from acute cases.

All six selected isolates for application of neutralization test on CAMS of ECEs gave positive results so it could be used for LSDV detection. Neutralization test is sensitive but is expensive, laborious and time consuming. Such results is inagreement with **House et al., (1990), Hassan et al., (1992), El-Bagoury et al., (1995), Ismael, (2000) Younis and Aboul-Soud, (2005) and Aly et al., (2006)** who used neutralization test for virus detection using cell cultures.

It was found that isolated LSDV posses no haemagglutination ability for bovine, sheep, chicken and rabbits RBCs. This finding is in harmony with **Uppal, (1963)** who mentioned that sheep poxvirus failed to agglutinate erythrocytes of goat, cattle and horse and **Shakya et al., (2004)** who stated that goat poxvirus did not agglutinate sheep, goat, pig, rabbit, dog, chicken and human type (O) erythrocytes.

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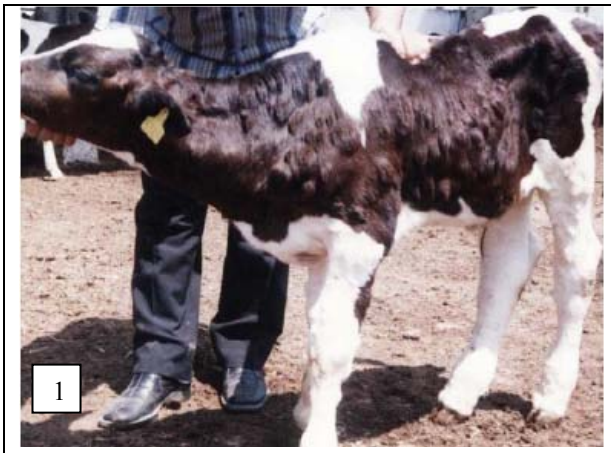
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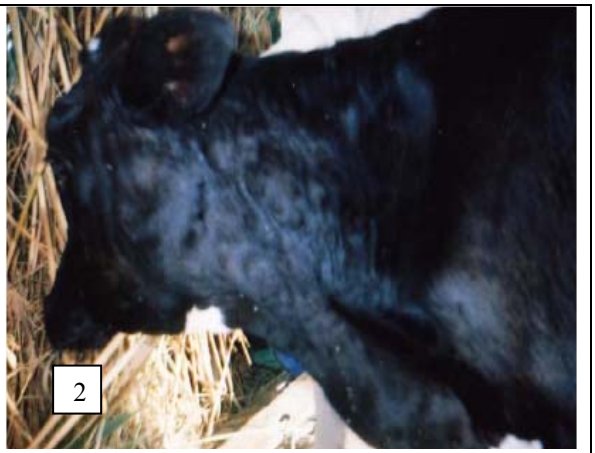
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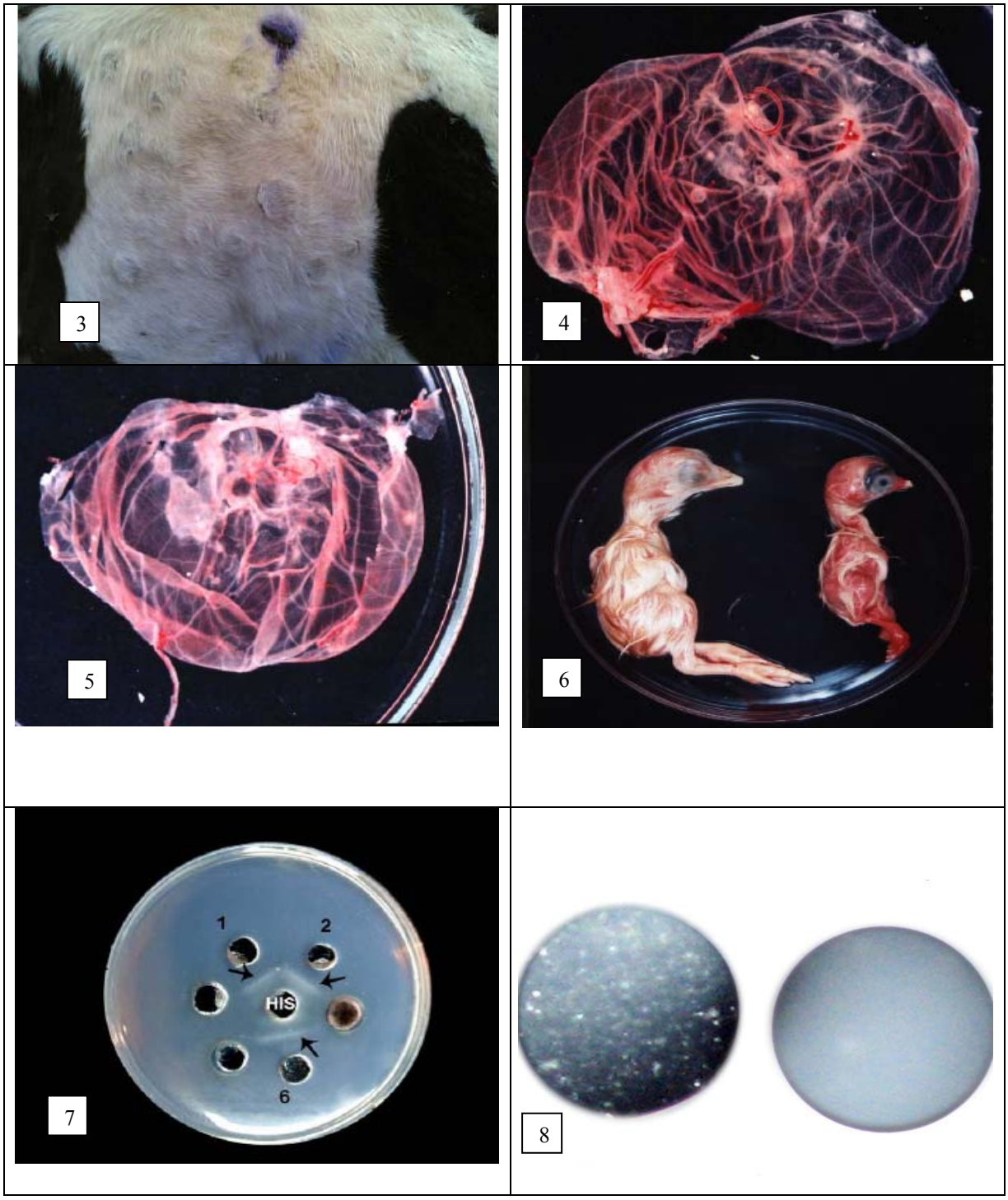

Figure (1) Skin nodules scattered all over the body of infected calf.
Figure (2) Skin nodules of LSD in adult cow.

Figure (3) Sit fasts lesions in infected calf.

Figure (4) Characteristic pock lesions on CAM infected with isolated virus from skin lesion on the fourth passage.

Figure (5) Characteristic pock lesions on CAM infected with isolated virus from lymph node on the fifth passage.

Figure (6) Chicken embryo inoculated with isolated virus (right) and uninfected control (left). Chicken embryos usually die within 5–7 days following inoculation. The infectedembryos shows multiple hemorrhages, oedema, stunted growth and abnormal feathering.

Figure (7) AGPT showing clear precipitin lines appeared between skin virus isolates (after 5th passage) and the prepared LSDV hyperimmune serum.

1, 2 and 6 = numbers of the virus isolates.

HIS= hyperimmune serum.

Figure (8) latex agglutination test (LAT): appearance of the latex agglutination indicate positive result(left) and absence of agglutination indicate negative result(right).

بقار على الغشاء اللقانقى الاعزل و تعريف فيروس مرض الجلد العقدى من مخصب دجاجالمشيمى لبيض

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مرض الجلد العقدى هو مرض فيروسى جلدى معدى يصيب الابقار و يسبب المرض خسارة اقتصادية فادحة. وقد أستهدفت هذة الدراسة محاولات لعزل فيروس مرض الجلد العقدى من أبقار مصابة ظهرت عليها عقد جلدية منتشرة على جسم الحيوان كله أو موجودة بشكل موضعى حيث تم تجميع عدد ٦٧ عينة من هذة الابقار بغرض عزل الفيروس بعدد٢٥ عينة من جلد ، ١٢ مسحة انفية ،١٢ عينة دم ، ٩ من الغدد الليمفاوية ، ٤ من الرئة ، ٣ من الكلية و٢ من الكبد. و قد تم حقن العينات بعد تحضيرهاعلى الغشاء اللقانقى المشيمى لبيض دجاج مخصب عمر ٩ أيام و تم اجراء خمس تمريرات لكل عينة. تم تحضير مصل نوعى من فيروس مرض الجلد العقدى (العترة الكينية).

تم التعرف على القيروس بأستخدام اختبار الترسيب فى الاجار، اختبار التلزن اللثى ، اختبار التلزن الدموى العكسى السلبى، اختبار التلزن الدموى السلبى و اختبار التعادل المصلى باستخدام المصل النوعى المحضر.ثم تم اختبار قدرة الفيروس المعزول على تلزن كرات الدم الحمراء المحضرة بنسبة ١% من الابقار، الدجاج ، الارانب و الاغنام.

وقد خلصت التجارب الى النتائج الاتية الفيروس المعزول من الابقار المصابة هو فيروس مرض الجلد العقدى واختبارى التلزن العكسى السلبى والتلزن اللثى أكثر حساسية للتعرف على الفيروس من اختبار الترسيب فى الاجار. يمكن استخدام اختبار التعادل المصلى فى الغشاء اللقانقى المشيمى لبيض الدجاج المخصب للكشف عن وجود الفيروس الا انه اختبار عالى التكلفة ويستهلك الوقت والجهد. الفيروس المعزول ليس لدية القدرة على تلزن كرات الدم الحمراء المحضرة من الابقار، الدجاج ، الارانب و الاغنام.