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Serodiagnosis of Lumpy Skin Disease Using Sheep Pox Virus Compared to a Commercial ELISA Kit

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ABSTRACT

Evaluation of the humoral immune response against Lumpy Skin Disease (LSD) in vaccinated and infected cattle is a very important issue for controlling it in Egypt. This study was conducted to evaluate and compare newly commercial ELISA kit and traditional virus neutralization test (VNT) using sheep pox virus (SPV) and Lumpy Skin Disease Virus (LSDV) for monitoring the humeral response against LSDV. Sensitivity and specificity of VNT were higher in case of using LSDV (96% and 100%, respectively) than in case of using SPV (89.3% and 98.6%, respectively) while they were 98.6% and 97.3%, respectively in case using of commercial ELISA Kit, which detected the highest number of positive samples (n =76) and (70 %) followed by VNT using LSDV (n =72) and (67.5 %) and finally VNT using SPV (n =68) and (67.5 %) for tested 150 control and 200 field samples respectively. The agreement between VNT using SPV and ELISA was achieved in (67) and (123) positive, (73) and (58) negative control and field samples, respectively, with overall proportion agreement (Po) as 0.93 and 0.90 with Kappa index of 0.86, and 0.78 for control and field samples respectively, while in case of using LSDV the agreement between VNT and ELISA was achieved in (72) and (134) positive, (74) and (59) negative control and field samples with overall proportion agreement of 0.97 and 0.96 with a Kappa index of 0.94 and 0.90 for control and field-tested samples respectively. Using hyperimmune sera prepared against LSD, the highest dilution gave positive results in the commercial ELISA kit and VNT using LSDV was 1/128 while it was 1/64 using SPV. This study illustrated that the VNT using LSDV is the most specific serological test for detecting LSD antibodies rather than ELISA commercial kit and VNT using SPV. Still, the test is not as sensitive enough as the commercial ELISA kit which was the most sensitive test for detection of LSD antibodies in vaccinated and infected cattle.

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INTRODUCTION

Lumpy skin disease (LSD) is a viral disease of cattle caused by the lumpy skin disease virus (LSDV) **(OIE, 2018).** LSDV is classified as a member of genus Capripoxvirus within a subfamily Chordopoxvirinae family Poxviridae together with goat pox (GP) and sheep pox (SP) viruses. Viruses of this genus share 97% of genomic nucleotide sequence identity, cross-protectivity; they are serologically indistinguishable as infection with one strain provides immunity against all other strains because of their homologous antigenicity. Thus single relative vaccine strain has been used to protect cattle, sheep and goats against Capri Poxviruses **(Tuppurainen *et al*., 2015** and **Al-Salihi. 2014).** In Egypt, LSDV was isolated and identified for the first time during a devastating outbreak in Suez and Ismailia governorates in 1989 **(House *et al*., 1990 and Davies, 1991)**. In early 2006, a severe LSD outbreak was introduced to Egypt by importing cattle from Ethiopia that affected the local bovine population in different governorates, causing severe economic

losses. The disease reappeared in 2012 till 2019, producing focal points of infection annually **(FAO-RNE, 2013, El-Haig 2017, Mona *et al*., 2019 and Adel, 2021).**

In Egypt, the Romanian SPV vaccine strain is being used to immunize bovine and small ruminants against Capri Poxviruses **(Tuppurainen *et al*., 2011 and Fatma *et al*., 2018).** Recently a live attenuated LSD (Nethling strain) vaccine has been introduced and authorized to vaccinate cattle against LSD in Egypt **(Adel, 2021 and *Nermin et al., 2021*).**

According to the Office International of Epizootic (OIE), its recommended for detection of LSD antibodies in sera of vaccinated cattle for vaccine evaluation by virus neutralization test (VNT) while Enzyme-Linked Immune Sorbent Assay (ELISA) is a suitable test for determining the herd and individual animals' status regarding LSDV infection **(OIE, 2018)**. The immune response of vaccinated cattle with SPV against LSD has been evaluated using VNT and ELISA **(Aboul Soud *et al*., 2004 and 2005, Ayatollah *et al*., 2015 and Yasser *et al*., 2015).**

Although VNT has been regarded as "the gold standard test" and the most specific test for detecting anti-LSD antibodies, it is not sensitive enough to identify contact animals and mild infected cases **(EFSA, 2015).** Whereas ELISA requires standardization of its reactant, ye it has some advantages regarding testing speed is easy to run for a large number of samples on one plate and is cost-effective **(Babiuk *et al*., 2009 and Bowden *et al*., 2009).** Virus Neutralization Test has the advantage of its higher specificity reached 100% than ELISA Kit which has the advantage of its higher sensitivity than VNT **(Milena, *et al*., 2019; Milovan *et al*., 2019 and Krešic *et al*., 2020´).**

The present study aimed to evaluate and compare the efficacy of VNT using homologous (LSDV) and heterologous (SPV) versus a commercial ELISA kit for serodiagnosis of LSDV in control and clinically suspected field sera. Besides, the specificity and sensitivity of these assays will be determined.

MATERIALS AND METHODS

1.Animal ethics:

Blood samples were collected from private cattle farms within the frame of monitoring for LSD under the Authority of the General Veterinary Services; Ministry of Agriculture. Good veterinary practice and animal welfare were considered during blood sampling and no formal certificate was required as this study did not include any animal experiments.

2.Viruses:

Live attenuated SPV (Romanian strain) with a titer 10 $^{5.5}$TCID $_{50}$/ml (propagated on Vero cell line) and live attenuated LSDV with a titer10 $^{5.5}$TCID $_{50}$/ml (propagated on MDBK cell line) were supplied by Pox Virus Research Department (PVRD), Veterinary Serum and Vaccine Research Institute (VSVRI) and used in VNT

3. Cell cultures:

African green monkey kidney cell line (Vero) used for VNT using SPV according to **Hosamani *et al*., (2004)** and **OIE, (2018)** and Madian Derby Kidney Cell Line (MDBK) used for VNT using LSDV according to **OIE, (2018)**; were supplied by PVRD. These cell lines were propagated and maintained using Earle's Minimum Essential Medium (MEM) supplied by Sigma Chemical Company, USA and used as growth medium supplemented with 10 % newborn calf serum and as maintenance medium with 2% newborn calf serum (Gibco Laboratories, New Zealand).

4. Serum samples:

From El-Fayoum Governorate; seventy-five (75) serum samples were collected from LSD unvaccinated diseased cattle with ages ranging from 6 up to 12 months come from unvaccinated dams in addition to seventy-five (75) (the diseased cases were confirmed using PCR), negative serum samples collected from different newborn calve serum batches used for tissue culture cultivation. In addition, 200 serum samples were collected from diseased and contact healthy cattle and (75) serum samples were collected from vaccinated 25 cattle with LSD vaccine (Neethling strain) as follow:

1- 25 samples after 2 weeks post-vaccination (WPV)
2- 25 samples after 3 WPV
3- 25 samples after 4 WPV

All serum samples were stored at -20°C till application of ELISA and VNT

5. Control positive and control negative serum samples:

Sheep Pox and LSD hyperimmune sera were prepared in rabbits according to the method described by **(OIE, 2018)** and as positive control and antibody-free newborn calf serum (Gibco Laboratories, New Zealand) was used as negative serum control was used in VNT.

6. Polymerase Chain Reaction (PCR)

Polymerase chain reactions were done on crust samples collected from 75 diseased cattle with LSD with different disease stages (positive control); the test was conducted according to (OIE, 2018) using primers developed for viral attachment protein gene. The size of the expected amplicon is 192 base pairs (bp). The primers have the following gene sequences:

Forward primer:
5"-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3"
Reverse primer:
5"-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3".

7. Serological tests

7.1.Virus neutralization test (VNT)

All 425 collected sera were screened against LSDV according to the **OIE, (2018)** method. The virus neutralization index (NI) was estimated according to **Cottral (1978).**

7.2.Commercial ELISA kit

A commercial ELISA kit ID Screen® Capripox Double Antigen Multi-species manufactured by ID Vet., (France) batch /N*de lot H35 was used to screen specific antibodies against LDSV. The procedure was conducted according to the instruction of the manufacturer and the optical density used for plate reading was 450 nm. The interpretations of the obtained results were based on the calculated S/P ratio (higher S/P ratio than 30% is considered a positive result for the presence of LSDV specific antibodies).

8. Statistical analysis:

To detain VNT using SPV and LSV, the results of True positive (TP); False positive (FP); True negative (TN) and False negative (FN) were determined using SPSS program version 25, 2021.

Specificity = TP / (TP + FN).

Sensitivity = TN / (TN + FP).

And to compare the performance of used tests, the Kappa test was used according to (**Thrusfield, 2007).**

	Test B	
Test A	+Ve	-Ve
+Ve	A	B
-Ve	E	D

The observed proportionate agreement (Po)

Po = a+d / a+b+c+d

The probability of random agreement (Pe)

Pe +Ve = a+b /a+b+c+d *a+c / a=b+c+d

Pe –Ve = b+d /a+b+c+d* c+d / a+b+c+d

Overall random agreement

Pe = Pe +Ve+Pe -Ve

Cohen's Kappa

K = Po –Pe /1-Pe

All statistical analysis and methods were done according to **Milena *et al*., (2019) and Krešić *et al*., (2020).**

RESULTS

1.Sensitivity and specificity of ELISA and VNT using negative and positive control samples:

One hundred and fifty serum samples were divided into 75 serum samples collected from cattle showing different symptoms and stages of LSD from mild to severe cases as shown in figure (1) and confirmed using PCR for detection of 192 bp, which is characteristic for LSDV as shown in figure (2) act as positive control LSD serum and 75 negatives control samples collected from different batches of bovine serum used for tissue culture propagation. The obtained results are shown in table (1) demonstrate the sensitivity and specificity of VNT using SPV for detection of LSDV antibodies as 89.3% and 98.6% respectively, while they were 96% and 100% respectively in using LSDV and in case of using ELISA kit; these values were 98.6% and 97.3%, respectively.

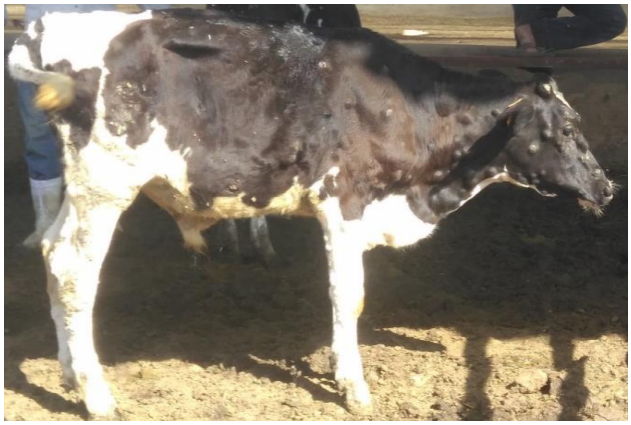

Fig. 1: generalized LD nodule in affected cattle.

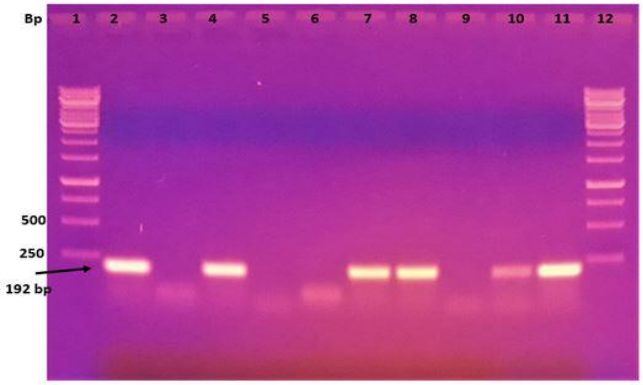

Fig. 2: Amplification of attachment protein gene of LSD showing fragment length of 192bp, lane (1) 1 Kb DNA ladder, lane (2) positive control, lane (4, 7, 8 10 and 11) samples collected from clinically infected cattle, lane (3, 5, 7 and 9) negative control samples.

Table 1: Sensitivity and specificity of VNT using SPV and LSDV compared to ELISA Kit using negative and positive control samples:

Test used	VNT using SPV	VNT using LSD	Commercial ELISA KIT
Sensitivity%	67/75 (89.3%)	72/75 (96%)	74/75 (98.6%)
Specificity%	74/75 (98.6%)	75/75 (100%)	73/75 (97.3%)

2. Comparative mean reactivity of VNT using SPV and LSDV compared to ELISA Kit for LSD prepared hyperimmune sera and vaccinated bovine sera:

By testing 75 serum samples weekly collected from 25 vaccinated calves, the results presented in table (2) show positive results for all serum samples by commercial ELISA KIT and VNT using LSDV along the period of study 2 weeks post-vaccination (WPV) till 4WPV with 100% positive while in case of VNT using SPV it was 80 % at 2WPV and 100 % at 3 and 4 WPV with overall 93%. As shown in tables (3) and (4), the antibody titers for serum collected from vaccinated calves estimated by VNT using SPV and LSDV and commercial ELISA kit were positive up to 1:64 while the use of LSD hyperimmune serum showed antibody titers estimated by ELISA kit and VNT using LSD and SPV at 1:128, 1:128 and 1:64 respectively.

Table 2: Detection of antibodies in vaccinated calves

Sample collection		VNT using SPV		VNT using LSD		Commercial ELISA KIT	
Time	No.	+Ve	+Ve %	+Ve	+Ve %	+Ve	+Ve %
2 WPV	25	20	80%	25	100%	25	100%
3WPV	25	25	100%	25	100%	25	100%
4WPV	25	25	100%	25	100%	25	100%
Total	75	70	93.3%	75	100%	75	100%

Table 3: Summary of comparative mean reactivity of LSD vaccinated bovine sera and prepared hyperimmune sera against LSD tested by VNT using SPV and LSDV compared to ELISA Kit.

Serum dilution	VNT NI using LSD	VNT NI using SPV	Commercial ELISA kit S/P ratio
Vaccinated calves' serum samples			
1:2	(+) 2.75	(+) 2.50	(+) 48
1:4	(+) 2.75	(+) 2.50	(+) 45
1:8	(+) 2.75	(+) 2.25	(+) 40
1:16	(+) 2.50	(+) 2.00	(+) 37
1:32	(+) 2.00	(+) 1.75	(+) 35
1:64	(+) 1.75	(+) 1.50	(+) 32
1:128	(-) 1.25	(-) 1.00	(+) 30
1:256	(-) 1.00	(-) 1.00	(-) 29
Prepared hyperimmune sera against LSD			
1:2	(+) 3.25	(+) 2.75	(+) 47
1:4	(+) 3.00	(+) 2.50	(+) 46
1:8	(+) 2.50	(+) 2.25	(+) 43
1:16	(+) 2.25	(+) 2.25	(+) 38
1:32	(+) 2.25	(+) 1.75	(+) 37
1:64	(+) 1.75	(+) 1.50	(+) 33
1:128	(+) 1.50	(-) 1.00	(+) 33
1:256	(-) 1.25	(-) 1.00	(-) 28

NB: NI >1.5 and S/P ≥ 30 consider positive results.

Table 4: Sensitivity of the VNT using SPV and LSDV as defined by the highest dilution giving positive test results in comparison with the commercial ELISA kit.

Serum sample	VNT using SPV	VNT using LSD	Commercial ELISA Kit
LSD vaccine	1:64	1:64	1:64
LSD hyperimmune sera	1:64	1:128	1:128

3. Comparison of the performance of the ELISA KIT and VNT for testing control samples by using SPV and LSDV:

The performances of VNT using SPV and LSDV were compared to that of ELISA on 150 samples in total. The highest number of positives was detected by ELISA (n 76), followed by VNT using LSDV (n 72) and VNT using SPV (n 68), as presented in table (5). The compatibility of results obtained by VNT using SPV and ELISA resulted in a Kappa index of 0.86 with an overall proportion agreement (Po) of 0.93. Agreement between VNT using SPV and ELISA was achieved in 67 positive and 73 negative samples. The compatibility of results obtained by VNT using LSD and ELISA was shown to result in a Kappa index of 0.94 with an overall proportion agreement of 0.97. Agreement between ELISA and VNT using LSDV was achieved in 72 positive and 74 negative samples.

Table 5: Percent of positive and negative samples tested by VNT using SPV and LSDV compared to ELISA Kit using negative and positive control samples:

Test used	VNT Using SPV	VNT using LSD	Commercial ELISA KIT
Positive percent	68/150 (45.33%	72/150 (48%)	76/150 (50.6%)
Negative percent	82/150 (54.66%	75/150 (50%)	74/150 (49.3%)

4. Comparison of the performance of the ELISA and VNT for testing field samples by using SPV and LSDV:

The performances of VNT using SPV and LSDV were compared to that of ELISA carried out on 200 unknown field samples of infected and vaccinated cattle in addition to healthy and contact cows in total. As shown in table (6), the percentage of positive samples and negative samples by ELISA were (70%) and (30%) respectively, while it was (67.5 %) and

(32.5%) by VNT using LSDV and (62.5%) and (37.5%) by VNT using SPV. The compatibility of ELISA kit and VNT using SPV resulted in a Kappa index of 0.78 with an overall proportion agreement (Po) of 0.90 as an agreement between VNT using SPV and ELISA was achieved 123 positive and 58 negative samples. The compatibility of the results obtained by VNT using LSD and ELISA resulted in a Kappa index of 0.90 with an overall proportion agreement of 0.96 as an agreement between VNT using LSD and ELISA was achieved in 134 positive and 59 negative samples.

Table 6: Percent of positive and negative samples tested by VNT using SPV and LSDV compared to ELISA Kit using field samples:

Test used	VNT using SPV	VNT using LSD	Commercial ELISA KIT
Positive percent	123/200 (61.5%)	135/200 (67.5%)	140/200 (70%)
Negative percent	75/200 (37.5%)	65/200 (32.5%)	60/200 (30%)

DISCUSSION

Recently, veterinary authorities in Egypt requested manufacturing a local live attenuated LSD vaccine based on the last successive outbreaks in Egypt from 2018 to date. Accordingly, we designed this endeavor to verify the efficacy of using heterologous (SPV) and homologous (LSDV) as virus-neutralizing for measuring seroconversion in bovines after vaccination as well as in sera from clinically suspected infected animals, compared to a commercial ELISA KIT as a standard approved test for detecting LSDV antibodies in vaccinated and infected animals.

Results of VNT for detection of LSDV antibodies revealed higher sensitivity (96%) and specificity (100%) using homologous LSD higher than that using SPV (89.3% and 98.6%, respectively). Despite the cross-neutralization between both viruses, the LSDV was superior to SPV in serodiagnosis of LSDV infected bovines due to the homogeneity similarity coming in agreement with the results which have been reported by **Abutarbush (2014); Molla *et al* ., (2017); Abutarbush and Tuppurainen (2018); Eman, (2018) ; Jihane *et al*., (2020) and Nermin *et al* ., (2021)** who have confirmed incomplete cross-neutralization between capripox viruses, yet protection of using one virus vaccine ranged from 70 to 80% protection at the field level.

The sensitivity was higher in case of using a commercial ELISA kit (98.6%) than that obtained by homologous VNT (96%) with lower specificity (97.3%). This finding was in accordance with **Babiuk *et al*., (2009); EFSA** (**2017); Milena *et al*., (2019) and Krešic´*et al*., (2020)** as VNT was the most specific serological test with specificity up to 100% but less sensitive (94-95%) than ID Vet commercial ELISA kit (96%) for detection LSD antibody in all contact animals. Although VNT has been considered the most specific serological method, it was not sensitive enough to detect seropositive contact animals as mild diseased or vaccinated animals might develop low levels of undetectable neutralizing antibody (**Tuppurainen *et al*., 2017).**

For more confirmation for the obtained sensitivity results, a comparison of mean reactivity of VNT using SPV and LSDV compared to ELISA Kit was made for LSD prepared and vaccinated bovine sera. Results presented in tables (2), (3) and (4) showed the highest dilution antibody titers in sera from LSD vaccinated cows was 1:64 resulting in a positive NI (>1.5) by VNT using SPV and LSD viruses and commercial ELISA kit with 100% detection for protective antibodies for all serum collected from vaccinated calves by commercial ELISA kit and VNT using LSD along the period of study (2 WPV till 4 WPV) while in case of VNT using SPV it was 80 % at 2WPV and 100 % at 3rd and 4th WPV with overall 93.3%. This decrease in antibody detection percent lower than VNT using LSD and commercial ELISA kit could be attributed to the lower sensitivity of VNT using SPV than LSDV and commercial ELISA which may lead to the incapability of the VNT using SPV to detect lower antibody titers against LSD at the beginning of immune response (first 2 weeks) post-vaccination as previously reported by **Milena *et al*., (2019) and Krešic´*et al*., (2020)**.

Also, the results of antibody detection in case of using the prepared hyperimmune sera were incompatible with the previous results as it was higher in VNT using LSD and commercial ELISA kit (1:128) than in VNT using SPV (1:64) coming in accordance with **Milovan *et al*., (2019**) who reported that the high sensitivity of commercial ELISA kit make it able to detect low level of antibody body in high dilutions, early and late immune response against LSD vaccination than the using of VNT as the beak of LSD antibodies in vaccinated calves at 21 days.

By testing 150 control samples, and 200 field samples, the performances of VNT using SPV and LSDV was compared to commercial ELISA kit, and the highest number of positive samples was detected

by ELISA followed by VNT using LSDV and SPV as presented in table (5) and (6). These results are in agreement with obtained results of the higher sensitivity of ELISA than VNT and the higher sensitivity of VNT using LSD than SPV as it reflects on the compatibility of ELISA kit and VNT using SPV resulted in a Kappa index of 0.86 and 0.78 for control and field samples ,with overall proportion agreement (Po) of 0.93 and 0.90, respectively which increased in case of compatibility of ELISA kit and VNT using LSD resulted in a Kappa index of 0.94 and 0.90 for control and Field samples, respectively with overall proportion agreement of 0.97 and 0.96, respectively that indicate the higher capability of commercial ELISA kit to detect LSD antibodies in diseased cattle than VNT using SPV and LSD and the higher compatibility and agreement of the results obtained by VNT using LSD to that of ELISA kit than that obtained by VNT using SPV.

Similar results were obtained by (**Milena *et al*., 2019)** when they compared compatibility of VNT using LSD and commercial ELISA kits using Kappa test by testing 325 serum samples of vaccinated, positive and negative serum samples as the obtained overall proportion of agreement was 0.97 with Kappa index of 0.91. Also, **Krešić *et al*., (2020)** evaluated the serological diagnostic test for antibody detection against LSD using 291 serum samples resulted in Kappa index of 0.83 with an overall proportional agreement of 0.93 between ELISA kit and VNT using LSD as the agreement was achieved in and 40 negative and 238 positive samples.

CONCLUSION

It was noted that VNT using heterologous SPV qualified for seroprevalence for revaccination with 98% specificity but its low sensitivity (89.3%) might hinder its use for diagnosis of infected or recently vaccinated calves with LSD while the use of homologous LSD VNT may have the better advance of more sensitivity (96%) than SPV for detection of diseased cattle with LSD and its superior specificity (100%) for detection of free LSD cattle especially in borders Quarantine and imported cattle while in case of ELISA kit by having the highest sensitivity percent (98.6%) it is a test of choice for measuring immune response especially early one in vaccinated cattle with LSD vaccine and in case of outbreaks especially for early and mild stages of LSD with the advantage of cost and time-efficient and analysis of a huge number of samples in broad range screening activities.

Declaration of Conflicting Interests

The authors revealed that there is no potential conflicts of interest.

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