COMPARATIVE DETECTION OF LUMPY SKIN DISEASE VIRUS (LSDV) USING TAQMAN REAL-TIME PCR AND CONVENTIONAL PCR

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ABSTRACT. Lumpy Skin Disease Virus (LSDV) is a contagious virus that affects cattle and has a considerable impact on animal health due to its potential for significant economic losses. The diagnosis of LSDV requires a fast, accurate, and feasible method in a veterinary laboratory to reduce the spread of the disease. The Polymerase Chain Reaction (PCR) has proven to be rapid and sensitive in detecting the DNA of the LSDV in different biological samples. This study aims to establish a PCR assay for the detection of LSDV and to compare the detection of this virus in clinical samples using the conventional PCR and Taqman real-time PCR. The primer or probe used is based on the OIE Terrestrial Manual 2018 Chapter 3.4.12 Lumpy Skin Disease. Detection of LSDV was conducted using PCR while the test specificity and sensitivity were determined using 121 clinical samples from cattle with clinical signs and highly suspected LSDV. The Taqman real-time PCR detection showed better sensitivity compared to conventional PCR when tested with a 10-fold dilution of the samples. While for specificity, the conventional PCR and Taqman real-time PCR are comparable. Among these 121 clinical samples, 69.42% (n=84) presented positive results by Taqman real-time PCR and 25.62 % (n=31) were detected as positive by conventional PCR. This study concludes that skin, scab, meat, and lymph nodes were better samples for Taqman real-time PCR with 100% positive results while whole blood in EDTA, nasal swab, and saliva swab samples gave more than 50% positive results. For conventional PCR, the best samples for LSDV identification were skin (100 %), scab (80.95%), and meat (42.85%). The results suggest that the Taqman real-time PCR technique serves as a better diagnostic tool of LSDV in cattle on a variety of samples. In conclusion, the use of Taqman real-time PCR for detection of LSDV is more accurate and robust, thus preferable for LSDV detection.

Keywords: Lumpy Skin Disease (LSDV), Taqman real-time PCR, conventional PCR

INTRODUCTION

Lumpy skin disease virus (LSDV) of cattle along with Sheep Pox virus (SPPV) and goat pox virus (GTPV), comprise the genus Capripoxvirus in the subfamily *Chordopoxvirinae*, family *Poxviridae* (Balinsky *et al*., 2008; Sharif & Ghorashi, 2010). LSDV is a subacute to acute "List A" OIE bovine illness, identified by extensive skin lesions and signs distinctive to generalized poxvirus diseases (Coetzer *et al*., 1994; El-Kholy *et al*., 2008). LSDV is transmitted by haematophagous vectors such as mosquito Aedes *aegypti*, ticks *Rhipicephalus appendiculatus*, horseflies *Haematopota spp.* and other *Stomoxys* species which facilitates the rapid spread of the virus in optimal climatic conditions (John *et al.*, 2021). However, Capripoxvirus strains that cause LSD in cattle do not infect sheep and goats naturally (Ireland & Binepal, 1998). The LSDV disease is important in terms of economic as the disease causes permanent skin damage, mass loss, decreased milk yield, and infertility (Tupurainen *et al*., 2005).

LSDV is a contagious pathogen, which spread over from the Middle East and Africa into south-east Europe, the Caucasus, Russia, since the past ten years and also to Asia more recently (Naveen *et al*., 2021; John *et al.*, 2021).

The spread of the disease recently to disease-free country shows the importance of controlling and eradicating its transmission (Fatemeh & Azizollah 2021). The first hotspot in South Asia region was Bangladesh where the first incident took place on 14 July 2019. Soon after, more LSDV cases have been subsequently reported in other countries such as India, Bhutan, China, Vietnam, Hong Kong, Nepal, and Myanmar (Moumita *et al.*, 2021).

The disease can be diagnosed early based on clinical signs. However, the clinically inappropriate or mild disease may cause difficulty to diagnose. Hence, rapid laboratory techniques are crucial for the detection of LSDV. When compared to conventional diagnostic techniques like virus isolation on cell culture, the PCR technique offers high sensitivity and specificity, rapid identification of the virus, and reliable results within hours. Furthermore, real-time PCR is a sensitive and reproducible technique that could replace conventional PCR in diagnostic routines (Paiva *et al.*, 2010). This research aims to establish a PCR assay for the detection of LSDV and to compare molecular detection by conventional PCR as well as Taqman real-time PCR.

MATERIALS AND METHODS

Samples

A Malaysian strain of LSDV from Veterinary Research Institute (VRI), Ipoh was used as a control and for analytical sensitivity in this study. A total of 121 clinical samples such as whole blood in EDTA, scab, nasal swab, saliva swab, meat, skin, pooled organ, and lymph node were obtained from cattle with clinical signs and highly suspected with LSDV. From July 2021 to August 2021, the samples have been submitted to Central Zone Veterinary Laboratory for the LSDV diagnostic workout.

DNA Extraction

DNA was extracted directly from 200 µl of whole blood in EDTA (n=37) and transport media containing nasal swab (n=35) or saliva swab (n=6). While for tissue samples, up to 20 mg tissues from these samples; scab (n=21), meat (n=7), skin (n=3), pooled organ (n=6), and lymph node (n=6) were homogenized with mortar and pestle in the presence of sterile sand prior to centrifugation at 3000 rpm for 10 minutes at 4 ^{o}C to sediment the cellular or tissue components and bacteria (if present). Afterwards, 200 µl of the supernatant were aspirated for DNA extraction. Apart from these 121 samples, specimens previously confirmed by sequencing (data not shown) as positive for African swine fever (ASF; n=3), Peste des petits ruminants (PPR; n=1), African horse sickness (AHS; n=3), Parvovirus (n=2), and Influenza A virus (n=3) were also subjected to nucleic acid extraction as negative controls for the PCR. The Indispin Pathogen Kit (Indical, Germany) was used to extract nucleic acid according to the manufacturer's instruction and eluted in 35 µl of elution buffer provided in the same kit. The extracted total nucleic acid was stored at -20 °C before they were further analysed. To determine the assays sensitivity, one sample that has been confirmed infected with LSD through nucleic acid sequencing (data not shown) was chosen and diluted in a triplicate of 10-fold dilution ranging from 50 ng/µl (100) to 0.05 pg/µl (10^{-6}) before subjected to amplification (Table 1).

Conventional PCR

The PCR primers were established from the viral attachment protein-encoding gene and have the following sequences: Forward primer 5'-d TTTCCTGATTTTTCTTACTAT-3', Reverse primer 5'-d AAATTATATACGTAAATAAC-3' (Ireland & Binepal 1998). The size of the amplicon is 192

bp. DNA amplification was performed in a final volume of 25 µl containing the following reagents from Quantinova Probe PCR (Qiagen, Germany): 12.5µl of 2x QuantiNova Probe PCR Master Mix, 1 µl 0.20 mM of each primer (IDT, Singapore), 5.5 µl of RNase Free Water, and 5 µl DNA sample.

The reaction was performed in Applied Biosystems SimpliAmp™ Thermal Cycler (ThermoFisher Scientific, USA). The program used was as follows: Initial denaturation for 2 minutes at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 58 °C for 30 seconds and primer extension at 72 °C for 30 seconds, ending with a final extension period at 72 °C for 5 minutes. The final product of amplification was analyzed using a 100 bp DNA ladder (Bioline, Germany) as a molecular marker on a 1.5 % agarose gel and stained with 5 % FloroSafe DNA stain (Axil Scientific, Singapore). Amplicons were visualized using a UV transilluminator (GE Healthcare, USA) at a wavelength of 590 nm. Positive reactions were confirmed according to size (192bp). The positive and negative controls were included in all PCR reactions performed.

Taqman real-time PCR

The quantitative real-time PCR (qPCR) Taqman assay amplified and detected an 89 bp region, which encodes the intracellular mature virion envelope protein P32 (vaccinia virus H3L homolog) within LSDV ORF074. Forward Primers CaPV-074F1 5'-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3' and Reverse Primers CaPV- 074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' were used with the Taqman probe CaPV 074P1 5'-6FAM-TGG CTC ATA GAT TTC CT-IABkFQ-3' (Bowden *et al*., 2008). DNA amplification was performed in a final volume of 20 µl containing the following reagents from Quantinova Probe PCR (Qiagen, Germany): 2x QuantiNova Probe PCR Master Mix 10 µl, 1.6 µl 0.10 mM of each primer, 0.5 µl 0.10 mM of probe (IDT, Singapore), 1.3 µl of RNase-free Water, and 5 µl DNA sample.

The reaction was performed in an Applied Biosystems QuantStudio 3 real-time PCR System (ThermoFisher Scientific, USA) using the following fast mode amplification program: 95°C for 2 minutes; 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The results were generated by determination of the threshold cycle (Ct), the fractional cycle number at which the change in the fluorescence of each reporter dyes passed a fixed threshold value set in the log (exponential) amplification phase. For each reaction, the baseline was regularly set between cycles 3 and 15. Clinical sample templates with Ct values less than 37 were considered positive. All samples were performed in triplicate. The positive and negative controls were included in all PCR reactions performed. Tubes containing only RNase-free water and not the DNA samples were used as non-template control in the PCR reactions.

RESULTS

Analytical sensitivity and specificity of conventional PCR and Taqman real-time PCR of LSDV detection

The analytical sensitivity of the conventional PCR and Taqman real-time PCR of LSDV has been shown by testing the titration series of LSDV DNA template (Table 1). The testing was performed in triplicate of ten-fold dilutions of the samples, using the conventional PCR and Taqman real-time PCR protocol. For Taqman real-time PCR, the assay was consistently able to detect as few as 5 pg/µl (10^{-4}) with a standard curve as depicted in Figure 1. While the lowest detection limit for the conventional PCR assay is 5 ng/µl (10^{-1}) (Figure 2). The specificity test conducted using Taqman real-time PCR and conventional PCR also demonstrated no cross-reactivity to other relevant viral pathogens in the laboratory (Table 2).

Table 1. Analytical sensitivity of Taqman real-time PCR (ABI QuantStudio 3) and conventional PCR (ABI SimpliAmp)

DNA Concentration	Replicates	Taqman real-time PCR LSDV Result				Conventional PCR LSDV Result
		Ct Value	Ct value mean	SD	Result	
50 ng/µl ($10^0 = 1$)	1	23.381	23.221	0.140	Positive	Positive
	2	23.163			Positive	Positive
	3	23.121			Positive	Positive
5 ng/µl (10^{-1})	1	26.609	26.577	0.046	Positive	Positive
	2	26.599			Positive	Positive
	3	26.524			Positive	Positive
0.5 ng/µl (10^{-2})	1	29.916	29.701	0.193	Positive	Negative
	2	29.647			Positive	Negative
	3	29.541			Positive	Negative
0.05 ng/µl (10^{-3})	1	33.443	32.868	0.507	Positive	Negative
	2	32.677			Positive	Negative
	3	32.485			Positive	Negative
5 pg/µl (10^{-4})	1	37.829	37.068	0.663	Positive	Negative
	2	36.760			Positive	Negative
	3	36.615			Positive	Negative
0.5 pg/µl (10^{-5})	1	Undetermined	No Ct Value	-	Negative	Negative
	2	Undetermined			Negative	Negative
	3	Undetermined			Negative	Negative
0.05 pg/µl (10^{-6})	1	Undetermined	No Ct Value	-	Negative	Negative
	2	Undetermined			Negative	Negative
	3	Undetermined			Negative	Negative

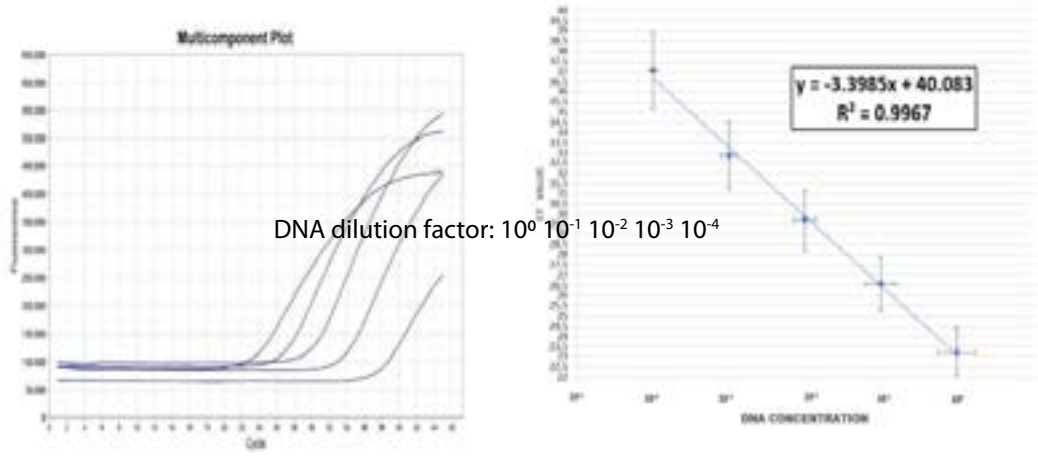

Figure 1. The results of Taqman real-time PCR to determine its sensitivity is demonstrated through a) multicomponent plots depicting the detection of a 10-fold dilution series containing LSDV DNA ranging from 50 ng/µl (10^0), 5 ng/µl (10^{-1} = 1), 0.5 ng/µl (10^{-2}), 0.05 ng/µl (10^{-3}), 5 pg/µl (10^{-4}), 0.5 pg/µl (10^{-5}) to 0.05 pg/µl (10^{-6}) per reaction, b) standard curve showing a linear relationship between LSDV DNA concentrations and Ct value, generated from mean data of experiments performed in triplicate

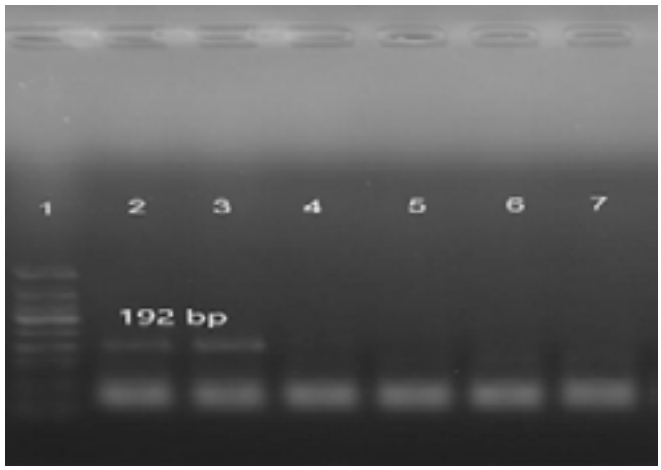

Figure 2. Result of the conventional PCR sensitivity test to determine the minimum detection limit: lane 1) 100 bp ladder, lane 2) LSDV DNA 50 ng/µl (10^0), lane 3) LSDV DNA 5 ng/µl (10^{-1}), lane 4) LSDV DNA 0.5 ng/µl (10^{-2}), lane 5) LSDV DNA 0.05 ng/µl (10^{-3}), lane 6) LSDV DNA 5 pg/µl (10^{-4}), and lane 7) LSDV DNA 0.5 pg/µl (10^{-5})

Table 2. Specificity test; determination of cross reactivity to other viral pathogens in the laboratory

Virus	Source	Taqman real-time PCR (mean Ct value)	Conventional PCR
African Swine Fever (ASF)	Central Zone Veterinary Laboratory	Undetermined	Negative
Peste des Petits Ruminants (PPR)	IDVet/Innovative Diagnostics (France)	Undetermined	Negative
African Horse Sickness (AHS)	Central Zone Veterinary Laboratory	Undetermined	Negative
Parvovirus	Central Zone Veterinary Laboratory	Undetermined	Negative
Influenza A Virus	Veterinary Research Institute	Undetermined	Negative

In addition to the specificity and sensitivity analysis, we were also able to compare the performance of both the Taqman real-time PCR assay and the conventional PCR in detecting LSD DNA across different sample matrices, namely the scab, skin, pooled organ, lymph node, meat, saliva swab, nasal swab, and whole blood in EDTA. The results are as tabulated in Table 3, where 69% of the 121 samples were detected positive by the Taqman real-time PCR assays in contrast to the 26% samples detected positive by the conventional assay. Samples that showed a 100% positive result of LSDV for Taqman real-time PCR were scab, meat, skin, and lymph node. Other samples, such as whole blood in EDTA, nasal swab, and saliva swab showed more than 50% positive results while pooled organs showed a result with the lowest positive rate of 33.33%. For conventional PCR assays, 100 % of the skin samples were detected positive,

Table 3. Detection of LSDV in cattle with clinical signs. Samples were tested using Taqman real-time PCR (ABI QuantStudio 3) and conventional PCR (ABI SimpliAmp PCR)

Type of sample	Number of samples	Taqman real-time PCR			Conventional PCR	
		Ct value (mean)	Positive	%	Positive	%
Whole Blood EDTA	37	32.368	21/37	56 .76	2/37	5.41
Scab	21	23.630	21/21	100	17/21	80.95
Nasal Swab	35	31.260	20/35	57.14	6/35	17.14
Saliva Swab	6	34.506	4/6	66.67	0/6	0
Meat	7	29.736	7/7	100	3/7	42.85
Skin	3	23.219	3/3	100	3/3	100
Pooled Organ	6	35.257	2/6	33.33	0/6	0
Lymph Node	6	34.618	6/6	100	0/6	0
Total	**121**	**30.574 (average)**	**84/121**	**69.42 (average)**	**31/121**	**25.62 (average)**

followed by 80.95 % for scab, and less than 50 % for the others. No positive results for saliva swab, pooled organ, and lymph node samples were produced.

DISCUSSION

The Taqman real-time PCR assay had a high analytical sensitivity with a lower limit of detection compared to conventional gel-based PCR, detected Capripox viruses isolated from worldwide outbreaks, and did not amplify DNA from related viruses in the Orthopoxvirus or Parapoxvirus genera (Stubbs *et al.*, 2011). This previous report coincides with the findings in this research where Taqman real-time PCR presented the best sensitivity results with low concentrations of LSDV DNA down to 5 pg/µl (fourth dilution) compared to conventional PCR which is only down to 5 ng/µl (second dilution) (Table 1).

Analytical specificity of the tests was also evaluated and no cross-reactivity to other relevant viral pathogens in the laboratory was detected using both Taqman real-time PCR and conventional PCR (Table 2). The primers and probes used in this study are specific for capripoxviruses including Lumpy Skin Disease which has been recommended by the OIE Terrestrial Manual 2018 and used worldwide. The specificity of the primers and probe used in Taqman real-time PCR was initially confirmed by performing a similarity search using the BLAST network service which indicated them as capripoxvirus-specific (Bowden *et al.*, 2008). Compared to the conventional PCR methods, the Taqman real-time PCR technique merged the highest test sensitivity with the highest test specificity and was, above all, not prone to PCR inhibition (Baric *et al.*, 2006).

In our study, Taqman real-time PCR assays detected more clinical samples as positive compared to conventional PCR (Table 3). The mean Ct values for saliva swab, pooled organ, and lymph node samples which were tested positive by the real-time assay and 100 % negative in the conventional PCR assay were higher than 34.5 suggesting that conventional PCR was unable to detect the virus DNA in these samples due to its slightly lower analytical sensitivity compared to the Taqman real-time PCR assays.

For the Taqman real-time PCR, the sample with the lowest average Ct value is the scab. Similarly, for conventional PCR, the highest positive results were also obtained from the skin and scab samples (Table 3). This finding correlates well with the observation that samples from nodular cutaneous lesions were more frequently positive by PCR and had higher concentrations of the virus than blood and pooled internal organ samples (Zeynalova *et al.*, 2016). Besides, this scenario could be due to the long period of LSDV shedding in the skin biopsies which occurs up to 92 days after infection (Tuppurainen *et al*., 2005). Particularly, our findings are consistent with a previous report by Ochwo *et al.* (2020) who concluded that skin nodule samples were preferable samples for PCR detection of LSDV than blood samples.

In our study, not all samples obtained from suspected clinical cases were tested positive by PCR. The negative results from blood samples taken from clinically symptomatic animals could be due to the short viraemic window which may have been missed. As found by Tuppurainen *et al.* (2005), the presence of LSDV in blood samples was detected positive by PCR in a brief period between 4 to 11 days after infection.

However, as reported by Tuppurainen *et al*. (2005), in comparison with LSDV detection in blood by PCR, detection of viraemia by virus isolation was able to produce positive results from the first day of infection until the twelfth day. Albeit the higher sensitivity, virus isolation

assay can be too time-consuming to use although this depends on how quickly a diagnosis must be confirmed. Especially during an outbreak, PCR is still the preferable option for a rapid diagnosis and a sufficiently sensitive method for detecting viral DNA. Besides, a real time PCR assay is able to detect viral DNA in skin lesions 53 days longer than virus isolation.

In addition, Taqman real-time PCR has the easiest and fastest testing process, involving minimal handling steps. Its disadvantage is the expensive cost of consumables and reagents, which is four times more than the standard PCR procedure. Although the cost of materials is high, the cost of personnel and disposal of hazardous waste will be much lower and more economical. Due to the simple testing procedure and the objective output of the results in the form of numerical data, the Taqman real-time PCR assay has a high potential for automation and appears to be the most suitable method for large-scale testing procedures (Baric *et al*., 2006).

CONCLUSION

In conclusion, Taqman real-time PCR assays described in this study are simple, sensitive, and specific thus enabling the testing of LSD to be rapid with high throughput. As the standard procedure for the detection of field LSDV strains in clinical samples, the Taqman real-time PCR assays is the best alternative method to replace the conventional gel-based PCR assays. Hence, Taqman real-time PCR is highly recommended to be used in conjunction with appropriate samples for preventing the rapid spreading of LSDV in Malaysia.

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