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APPLICATION OF REAL TIME - PCR FOR IDENTIFICATION OF SHEEP POXVIRUS, CAMEL POXVIRUS AND CONTAGIOUS ECTHYMA VIRUS ISOLATES

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SUMMARY

Recently, attention has been directed toward the application of Real time - PCR assays as a rapid and accurate tools for identification of Capripox, Parapox and Orthopox viruses that cause devastating diseases in farm animals in the Kingdom of Saudi Arabia.

SYBR Green (Real time ñ PCR assays with primer pairs; Capri - Ks.1 of Capripoxviruses, 045 Orf of Parapoxviruses and Q Orf of Panparapoxviruses were adopted on a panel of Saudi field isolates and reference strains of sheep and goat poxviruses, camel poxvirus, reference strains of Lumpy skin disease and vaccinia viruses, and Saudi field isolates of contagious ecthyma (Orf) virus. Capri ñ KS.1 primer set succeeded to amplify all test DNAs of sheep and goat pox, Camel pox, Lumpy skin disease and vaccinia viruses. With melting curve analysis, temperature of melting (Tm) scored by sheep and goat poxvirus, camel pox virus isolates and their reference strains were relatively identical (between 81.1 and 81.8OC), while Tm scored by Lumpy skin disease virus and vaccinia virus were 82.1 and 83.2OC respectively. No amplification was detected from DNAs of Orf virus isolates tested by Capri - KS.1 primers. However, the Orf virus isolates were fairly equivalent amplified with both of 045 Orf primer set (mean Tm score 92.5°C) and Q Orf primer set (mean Tm score 84°C), as well as with TaqMan Real time - PCR in the presence of TaqMan probe and Q Orf primer set. Otherwise, occasional nonspecific amplification of some isolates and

strains of Capripox and Orthopox viruses were detected by too late cycle of amplification (> 35 cycle) with 045 Orf and Q Orf primer sets.

INTRODUCTION

Sheep pox, contagious ecthyma and camel pox are the most important pox virus infection in sheep, goats and camels in Saudi Arabia. Traditional laboratory diagnosis of these diseases is based on the serological tests (Fluorescent antibody technique and virus neutralization test). Antigenic cross reactions between sheep poxvirus and contagious ecthyma (Orf) virus with inavilability of specific monoclonal antibodies can not always be avoided in the diagnostic serological test, Carn, 1995.

Conventional Polymerasee Chain Reaction (PCR) was developed for identification of the sheep poxvirus isolates by Mangana ñ Vougiouka et al, 1999 and Markoulatos et al, 2000. Also, performing of conventional and Real time ñ PCR was successed to identify contagious ecthyma virus Gallina et al, 2006 and Kottaridi et al, 2006. However, we have no available data on application of PCR assays in identification of Camel poxvirus.

The purpose of this study was the application of Real time ñ PCR assays in identification of Saudi field isolates of sheep pox, contagiuos ecthyma (Orf) and camel pox viruses in Riyadh Veterinary Diagnostic laboratory in matching with reference strains of Capripox and Orthopox viruses.

MATERIAL AND METHODS

MATERIAL

Virus	Strain/Isolate	Sample	Source/Reference
Sheep / goat pox	Kenyan strain (KSG0240)	Infected Vero cell cultures	National Research Center of Agriculture and Animal Resource (NRCAA), Riyadh
Sheep pox	Vaccinal, Romanian strain	Infected Vero cell cultures	Veterinary Vaccines Production Center (VVPC), Riyadh
Sheep pox	Isolate 382/24/04	Infected Vero cell cultures	Riyadh Veterinary Diagnostic laboratory (RVDL)
Sheep pox	Isolate 720/28/07	Skin lesion scraping	RVDL
ORF	Isolate 617/25/07	Skin lesion scraping collected from goat	RVDL

ORF	Isolate Al-Quassem 28/07	Skin lesion scraping collected from goat	RVDL
ORF	Isolate 77/29/08	Skin lesion scraping collected from sheep	RVDL
ORF	Isolate 167/29/08	Skin lesion scraping collected from sheep	RVDL
Camel pox	Vaccinal, Jouf- 78 strain	Infected VERO cell culture	NRCAA / Hafez et al,1991
Camel pox	Isolate U.04	Infected VERO cell culture	RVDL
Camel pox	Isolate 433/28/07	Infected VERO cell culture	RVDL
Lumpy skin disease	Egyptian Ismailia strain 1988	Infected MDBK cell culture	Veterinary Serum and Vaccine Research Institute, Cairo, Egypt / House et al, 1990
Vaccinia	Vaccinal, Elstree strain	Infected camel kidney cell culture	NRCAA / Hafez et al,1991

Primers:

The listed primer sets for Capripoxvirus and contagious ecthyma (Orf) virus manufactured by TIB – MOL Biol syntheselabor Gmb H Berlin, Germany were used in application of Real time – PCR assays.

Primer set	nucleotide sequence	Length (base)	Tm	Amplicon size (bp)	Reference
Capripox (Ks.1 5/Ks.1.6)	F 5`- gtgtgactttcctgccgaat 3`	20	60	149	*Gershon et al, 1989 - Mangana-Vougiouka et al, 1999*
	P_2 5` - tctattttatttcgtatatc 3`	20	48		
045 Orf	F 5` - cctacttctcggagttcagc 3`	20	62	250	*Kottaridi et al, 2006*
	R 5` gcagcacttctcctcgtag 3`	19	60		
Q Orf	F- 5` cagcagagccgcgtgaa 3`	17	56	170	*Gallina et al, 2006*
	R- 5` catgaaccgctacaacaccttct 3`	23	68		

Viruses:

The following list of Saudi field isolates and reference strains of Capripox, Parapox and Orthopox viruses was tested:

Viral DNA Extraction:

Viral DNA was extracted from clarified supernatant of skin lesion scraping Homogenates or infected cell cultures centrifuged at 3000 RPM for 10 min, using automated Mag NA pure Compact extraction machine (Roche) and Mag NA pure Compact Nucleic Acid isolation Kit I (Roche) Cat. No.: 03 730 964 001. The sample volume was 400 ul, and the elution volume was 100 ul. The extraction procedures was carried out following the manufacture's instruction, and the extracted DNA was kept at -20°C.

Real time - PCR techniques:

SYBR Green (Real time - PCR

Real time - PCR in the presence of SYBR Green (was performed on extracted DNA using Light Cycler 2.0 (Roche) using, one primer set for Capripoxvirus (Capri ñ Ks.1) and two different primer sets (045 Orf and Q Orf) for contagious ecthyma (Orf) virus, and Light Cycler Fast Start DNA master plus SYBR Green (kit (Roche) Cat. No. 03 515 889 001.

PCR assay was performed in a final volume of 20 ul reaction mix containing 9 ul water (PCR grade), 4 ul master mix 5x conc., 1 ul of each 10uM forward and reverse primer (final conc. of 0.5 uM each primer), and 5 ul of DNA (diluted 1 : 10 in PCR grade water). Cycling parameters were the following; 10 min at 95°C for pre- incubation followed by 30 or 40 cycles of denaturation a 95°C for 10 s, annealing at 50°C for 5 s in case of Capripox primer set and at 62°C for 5 s in case of contagious ecthyma (Orf) primer sets (045 Orf and Q Orf), and extension at 72°C for 15 s, then one cycle of melting and as described in the backñinsert of the kit. During the melting cycle, the temperature was increased by increments of 0.1OC / s between 65°Ch and 95°C.

TaqMan Real time - PCR

Real time - PCR in the presence of contagious ecthyma virus Q Orf TaqMan probe (5` 6 FAM ñ caccttcggctccac - BBQ) and Q Orf primer set was performed on extracted DNAs using Light Cycler Fast Start DNA master plus Hybo - probe kit (Roche) Cat. No. 03 515 575 001. PCR assay was performed in a final volume of 20 ul of reaction mix containing 8.5 ul PCR grade water, 4 ul master mix, 1 ul of each 10 um forward and reverse primer of Q Orf primer set and 0.5 ul of 10 uM Q Orf probe (final conc., of 0.25 uM), and 5 ul DNA (diluted 1 : 10 in PCR grade water). Cycling parameters were as follows, 10 min at 95°C for pre - incubation, followed by 40 cycles of denaturation at 95°C for 10 s, annealing and extension at 60°C for 35 s, then one cycle of cooling at 40°C as mentioned by Gallina et al, 2006.

Channel 530 qualitative detection and tempera-

ture of melting types of analysis were used in SYBR Green (real time - PCR assay, and channel 430 qualitative detection type of analysis was used in TaqMan real time - PCR assay.

For optimization of the assays at least 3 runs were done for each primer set with the extracted DNAs.

RESULTS AND DISCUSSION

All viral DNAs were tested by SYBR Green (Real time - PCR with Capri KS.1 primer set of Capripoxvirus and 045 primer set of contagious ecthyma (Orf) virus. Saudi field isolates of sheep pox and camel pox viruses either in skin lesion scrapings (crude sample) or in cultures of vero cells inoculated with the isolates as well as their reference strains were amplified with Capri KS.1 primer set gave Cross Point (CP) values between the 17^{th} and 25^{th} cycle of amplification, and showed peaks of melting temperature (Tm) between 81.1°C and 81.8°C (only one peak for each virus). Also Lumpy skin disease virus and vaccinia virus were amplified by Capri - KS.1 primers at cycles 30 and 38 with peak Tm of 82.1°C and 83.2°C respectively as shown in Fig (1). No amplification was detected from extracted DNAs of contagious ecthyma virus (Orf) isolates and non infected vero cell culture with Capri-KS.1 primer pair. Sheep and goat poxviruses and Lumpy skin disease virus are antigenically and genetically related members of the genus Capripoxvirus belongs to the Poxiviridae family. Melting curve analysis can distinguish products of the same length but of different G/C / A/T ratio that differed in less than 2OC Ririe et al, 1997. The variation in the Tm scores recorded by amplification products of sheep and goat pox viruses and Lumpy skin disease virus DNAs are directly related to the nucleotide sequence variations of the amplicons initiated by the used primers. The identical results of amplification platues, and peaks of Tm obtained through the use of Capri - KS.1 primer pair for amplification of extracted DNAs of the field isolates and reference strains of sheep poxvirus and camel poxvirus were somewhat unexpected. The reality of these results could be explained by Mercer, et al, 2002 who mentioned that most of essential and conservative genes are located in the central part of the genomes of poxviruses while the most of nonessential and variable genes are located in the terminal ends. The nucleotide sequence analysis of the two centrally located segments of Capripoxvirus DNA have shown a very similar gene orientation to that of the corresponding segments of vaccinia virus DNA (Gershon, et al, 1989), and camel poxvirus isolates were found to be related to vaccinia - variola viruses and classified as a member of genus Orthopoxvirus (Pandy, et al, 1985). Unfortunately no data is available concerning the application of real time - PCR for identification of Capripoxviruses as well among the use of PCR assay in diagnosis of camel pox to evaluate the present results.

Saudi field isolates of contagious ecthyma (Orf) virus infection in both sheep and goats gave amplification with primer pair (045 F and 045 R) of Orf virus between the 14th and 27th cycle of amplification, and Tm scores between 92.5 and 93.1OC (Fig. 2).

Also, solitary peak of Tm for each isolate indicates the formation of a single PCR product with no artifacts such as nonspecific amplification or primer dimmer. The primer 045 F and 045 R were chosen by Delhon, et al, 2004 for amplification of the highly conserved gene 045 coding for the late transcription factor VLTF - 1 of Orf virus isolate OV ñ SA00 (fragment of 392 bp) by conventional PCR. Nonspecific amplifications were occasionally obtained from DNA samples of some tested isolates and strains of Capripoxvirus and Orthopoxvirus with 045 primer set of Orf virus by too cycle of amplificatio (>35 cycle) with Tm scores (<90°C) (results not virus by too late cycles of amplification of poxivirus other than Orf virus by 045 IOrf primers, the high Tm score (≥92.5) of Orf virus isolates is characteristic and distinguishable, this high Tm score could be due to the length (392bp) and high G + C content of the target amplicon. Parapoxvirus genes is distinguished from other poxvirus genera by the high G + C content (approximately 64%) of the genome Delhon, et al, 2004.

On the other hand, real time - PCR assay based on the use of a minor groove binding (MGB) TaqMan probe and relies on the amplification of a 70 bp fragment from the conserved region of the Orf virus B2L gene that induces the major envelope protein was applied for testing of Saudi field isolates of Orf virus in presence of Q Orf primer set. Isolates were successfully amplified by the cycle ≤20th (only two isolates data is present in the available print as shown in Fig.3).

Also, amplification from DNA samples of some isolates and strains of Capripox and Orthpoxviruses were recorded by too late cycles (>35 cycles). The maximum number of 30 cycles is recommended to be used in such run to overcome these undistinguishable amplification where the melting curve analysis can not be done in TaqMan real time - PCR and the crusted scab lesions of Orf mostly contain high concentration of virus particles. On the other hand, using of Q Orf primer pair alone in application of SYBR Green (real time ñ PCR on all tested DNAs has been resulted in the same amplification that gained by TaqMan probe real time PCR, and the scored mean Tm was (84°C). The mean Tm scored by contagious ecthyma virus isolates with Q Orf primers SYBR Green real time - PCR was 83°C, (Gallina, et al 2006). However, one of the advantages of rea time - PCR is its quantitative capability, ten fol dilutions of the harvested viral DNAs of certai isolates and strains were made in water PC grade and tested. Based on SYBR Green I an TaqMan real time - PCR assays with the variou

tests dilution, every 1 : 10 dilution of DNA delayed the cross point (CP) value of amplification by approximately three cycles; this could be used to estimate the virus DNA concentration in each sample.

In conclusion, the data described in this study demonstrate that; SYBR Green I real time ñ PCR with Capri ñ KS.1 primer set can detect sheep and goat poxviruses, Lumpy skin disease virus and could differentiate them from contagious ecthyma (Orf) viruses of sheep and goats; the Capri ñ KS.1 primers could be used for identification of

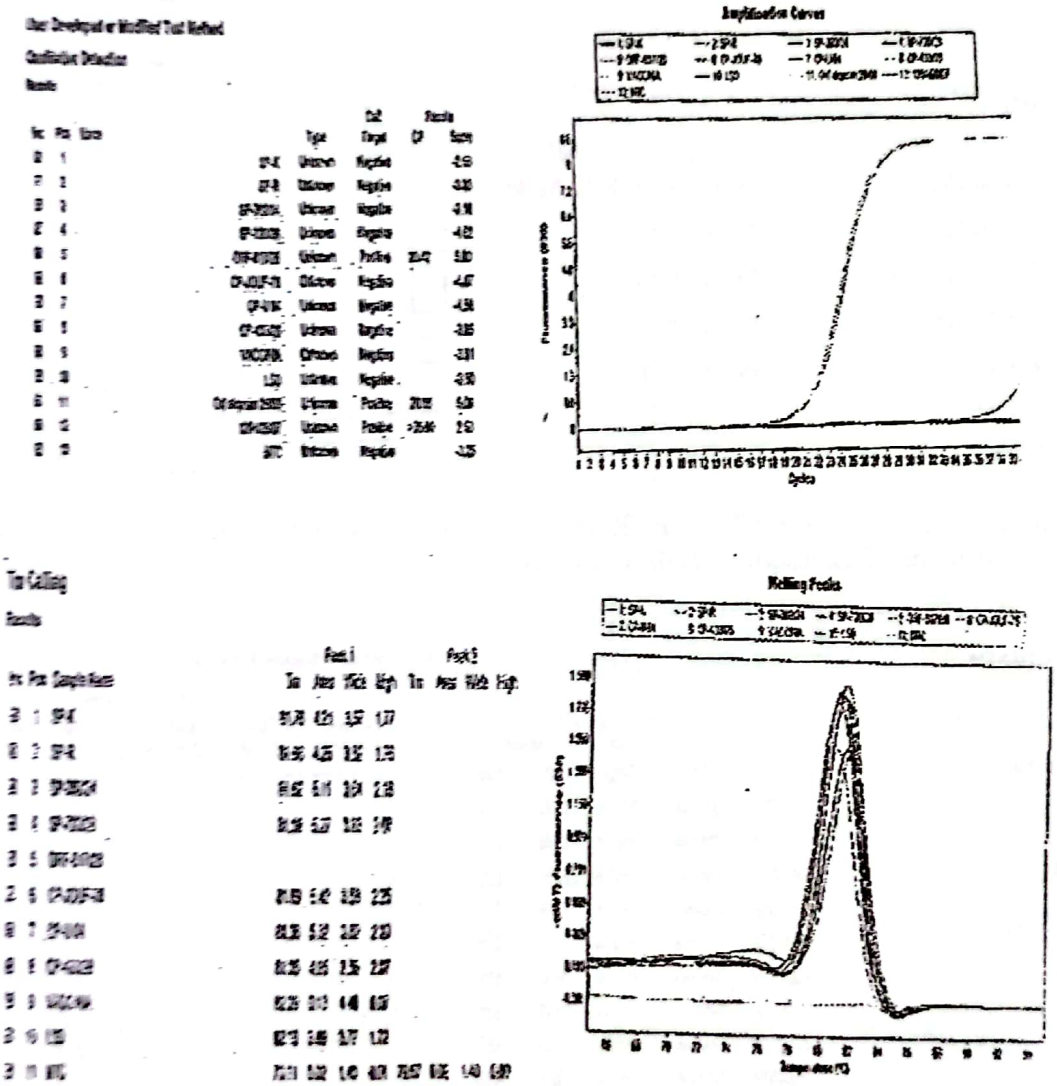

Fig. (1) : Results of Real Time PCR using Capri -KS.1 primer set in identification of Capripox and Orthopox virus strains and isolates.

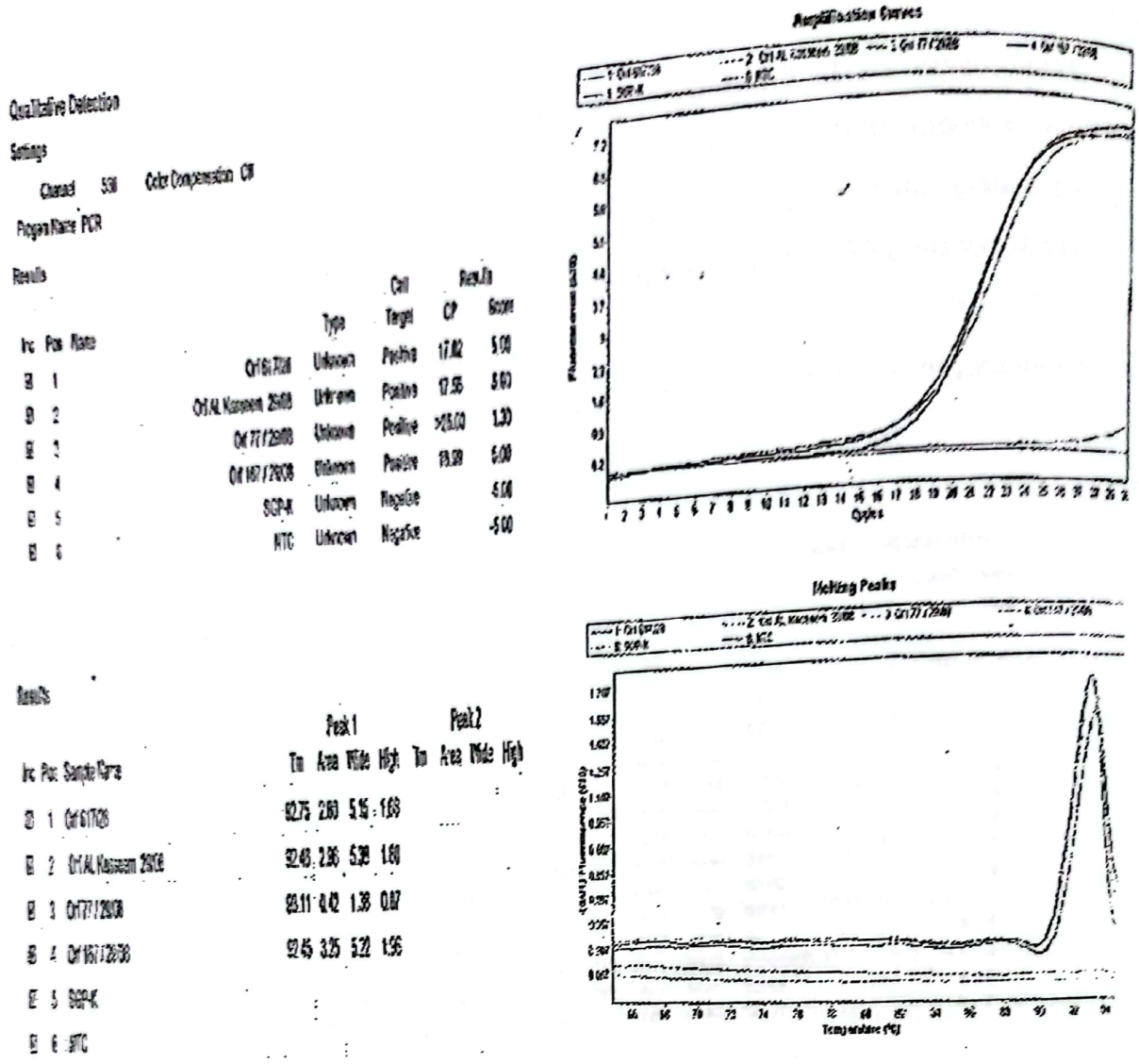

Fig. (2): Results of Real Time PCR using Orf 045 primer set in identification off Saudi field isolates of Contagious ecthyma virus.

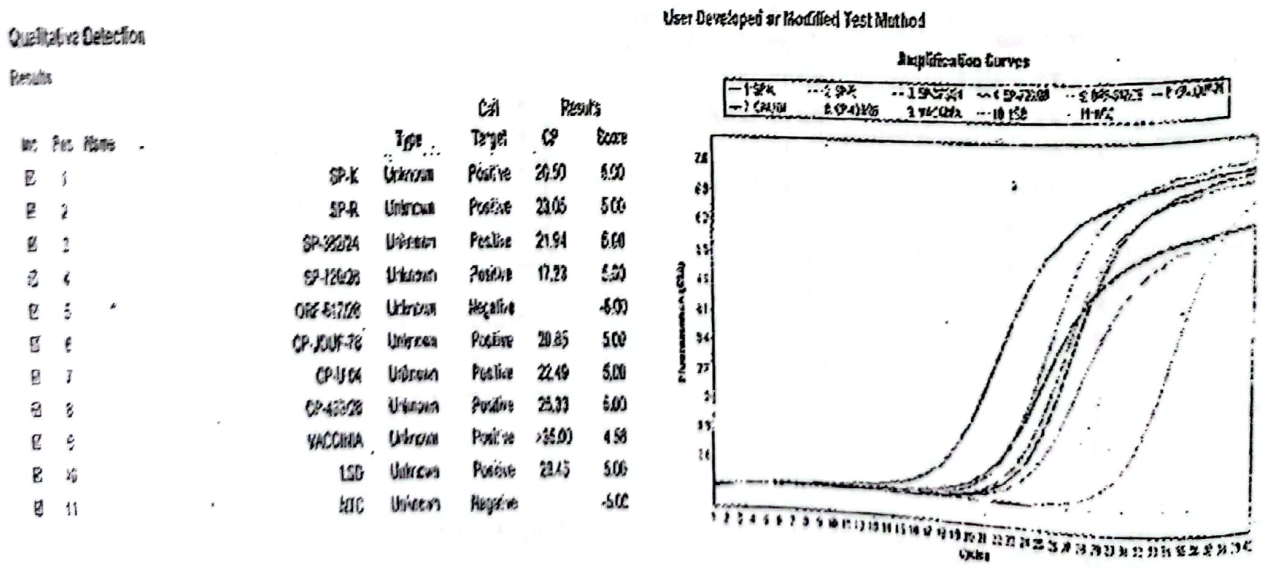

Fig. (3): Results of Real Time - PCR using of Q Orf TaqMan probe in identification of Saudi field isolates of contagious ecthyma virus.

camel poxvirus isolates; and SYBR Green I real time - PCR initiated with 045 Orf primer pair is a reliable assay for detection and differentiation of Orf virus isolates in the field samples.

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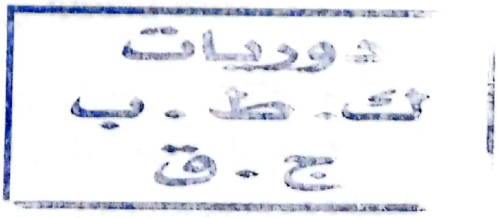

تطبيق إستخدام إختبار التفاعل المتسلسل لإنزيم البلمرة (الوقت الحقيقى) فى تعريف فيروسات جدرى الأغنام وجدرى الإبل وإلتهاب الجلد البثرى الوبائى

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*- المعمل المركزى للرقابة على المستحضرات البيطرية
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حديثاً نوجه الإهتمام إلى إستخدام إختبار التفاعل المتسلسل لإنزيم البلمرة (اللوقت اللحقيقى) كأدوات معملية دقيقة وسريعة فى تعريف فيروسات جدرى اللماعز ونظير الجدرى الحقيقى تلك التى تتسبب فى إحداث أمراض هامة فى حيوانات المزرعة بالمملكة العربية السعودية.

يشتمل هذا البحث على شرح لإستخدام إختبار التعاقل المتسلسل لإنزيم البلمرة (الوقت الحقيقى) بواسطة مادة الفلوروسين ***SYBR Green I*** وبادئ التفاعل الخاص بفيروسات جدري الماعز ***Capri – Ks.1*** والبادئات الخاصة بفيروسات نظير الجدري ***045 orf*** و ***Q orf*** وذلك للكشف عن معزولات وعترات فيروسات جدري الأغنام والماعز وجدري الإبل وإلتهاب الجلد البثري الوبائي وعترتين مرجعيتين لفيروس مرض الجلد العقدي ولقاح الجدري الآدمي. ولقد نجح الإختبار باستخدام بادئ فيروسات جدري الماعز في الكشف نوعيا على عترات ومعزولات فيروسات جدري الأغنام والماعز وجدري الإبل ومرض الجلد العقدي وفيروس لقاح الجدري الآدمي، وبتحليل منحنى الإذابة لنواتج التكبير في الإختبار كانت الإذابة القصوى المسجلة بواسطة عترات ومعزولات فيروسات جدري الأغنام والماعز وجدري الإبل متماثلة تقريبا عند درجات حرارة بين ٨١.١ °م إلى ٨١.٨ °م كما سجلت عترتي مرض الجلد العقدي ولقاح الجدري الآدمي درجة إذابة قصوى عند درجتي حرارة بين ٨٢.١ °م إلى ٨٣.٢ °م على التوالي، ولم يحدث تفاعل إيجابي لأي من معزولات فيروس إلتهاب الجلد البثري الوبائي وذلك باستخدام بادئ فيروسات جدري الماعز لكنها أعطت جميعها نتيجة إيجابية وبصورة متساوية تقريبا للإختبار باستخدام بادئي الفيروس ***Q orf , 045 orf*** وكانت الإذابة القصوى المسجلة لها عند متوسط درجتي حرارة بقيم ٩٢.٥ °م و٨٤ °م على التوالي وكذلك باستخدام البروب النوعي لفيروسات نظير الجدري ***TagMan probe***.

من ناحية أخرى فقد أظهرت بعض من عترات ومعزولات فيروسات جدري الماعز والجدري الحقيقي ناتج تكبيري غير نوعي في الإختبار باستخدام بادئ فيروسات نظير الجدري وذلك في المراحل الأخيرة من التكبير (بعد الدورة الخامسة والثلاثون) لكنها مختلفة فيما بينها في درجات حرارة الإذابة القصوى وبينها وبين درجات الحرارة المسجلة بواسطة معزولات فيروس إلتهاب الجلد البثري الوبائي.