Rapid Detection of Sheep Contagious ecthyma (ORF virus) in Al-Diwanyia Province by Real-Time PCR technique

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Abstract

Contagious ecthyma is a highly contagious, zoonotic, viral skin disease that affects sheep, goats and some other domestic and wild ruminants. Clinical diagnosis is often possible, but laboratory diagnosis is sometimes necessary. Serological methods exist, but electron microscopy is the most commonly used method. However, the lack of electron microscope by many veterinary diagnostic laboratories and the high cost. Therefore a practical and reliable method for diagnosis of parapoxvirus infection is required. The development of Real-Time PCR method for the Rapid molecular detection of parapoxvirus DNA has been specific and sensitive laboratory diagnosis tool for contagious ecthyma disease. Real-Time PCR was carried out by designing specific primers for amplified highly conserved region (GIF) gene ORF virus with an amplicon size of 147bp, which encoded using GM-CSF/IL-2 inhibition factor. The results showed there was specific and sensitive detection of ORF virus in sheep lip scrape tissue samples at (21 samples out of 23 clinical cases samples) in percent (91%). We concluded that Real-Time PCR technique provides a rapid and sensitive method for specific direct detection of contagious ecthyma ORF virus in sheep.

Key word: Contagious ecthyma, Orf virus, Sheep, Real-Time PCR.

Introduction

Contagious ecthyma also known as Sore mouth is highly contagious disease primarily of domestic sheep and goats. The disease is commonly

termed as (Orf) is also known as contagious pustular dermatitis and scabby mouth (1). Orf vir us is a member of parapoxviruses genus belongs to the family poxviridae. This genus also includes pseudocowpox virus (PCPV) and bovine papular stomatitis virus (BPSV) in cattle, squirrel parapoxvirus (SPPV), and parapoxvirus of red deer in New Zealand (PVNZ) (2, 3). ORF virus are ovoid in shape, with a crisscross patterned tubule-like structure on the particle surface and has viral genome consist of linear double strand DNA (4). Orf virus causes zoonotic disease, and occurs worldwide causing high morbidity in sheep and goats (5). Young animals are the most susceptible to occurrence of disease with significant productivity losse, mortality ranges from 10% t 90% in lambs and kids (6). The classical clinical disease is usually manifested as scabby lesions around the mouth (7). The lesions are initially erythematous spots or swelling, and followed by formation of papules which become bigger and pustules with a yellowish creamy appearance, these papules progress to develop into scabs (8). Although it is easy to diagnose the disease, the lesions and symptoms of ORF disease confused with other skin diseases require laboratory confirmation, which includes serological and molecular techniques, or electron microscope examination (9, 10). Regarding highly cost of electron microscope in isolation of ORF virus. Therefore, in this study, we developed a rapid, sensitive SYBR green-based real-time PCR molecular technique targeting GM-CSF/IL-2 inhibition factor, a highly conserved region of GIF gene of the ORFV, has been developed for the detection and of ORF virus in lip scraps skin lesions of small sheep.

Materials and Methods

Samples collection: 23 Samples of lip scrape tissue were collected from clinical examined lambs infected with Orf virus from different regions of Al-Diwanyia province. The samples were collected in sterile containers under aseptic conditions and transported as soon as possible to laboratory and stored in -20°C refrigerator until use for viral genomic DNA extraction.

Viral genomic DNA extraction: Viral genomic DNA was extracted from lip scrape tissue by using (Genomic DNA extraction tissue kit. Geneaid. USA). 200mg lip scrape tissues was placed in 1.5 ml microcentrifuge tube and homogenized in Tissue lysis buffer by using micropestle provided with the kit. Then, viral DNA was extracted according to kit instructions. The purified DNA was eluted in elution buffer provided with kit and store at -20°C, then used for preparation of Real-Time PCR master mix reaction. The extracted viral DNA was checked by Nanodrop spectrophotometer.

Real-Time PCR

SYBR green-based Real-Time PCR technique was conducted for rapid detection of ORF virus according to method described by Venkatesan (11). Real-Time PCR primers were designed by this study using conserved region (GIF) gene ORF virus (NCBI-GenBank Code: AF192803.1) with an amplicon size of 147bp, which encoded using GM-CSF/IL-2 inhibition factor. These primers were provided by Bioneer Company. Korea as showed in following table:

Primer	Sequence		Amplicon
GIF	F	**TCAACTGCGGCTTCTTCAAC**	147bp
	R	**GCGTTTCGTTTTCGTACTCC**	

The Real-Time PCR amplification reaction was done by using (AccuPower^{TM} 2X Green star qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table:

qPCR master mix	Volume
Viral Genomic DNA template	2.5µL
2X Green star master mix	25µL
GIF Forward primer (10pmol)	1µL
GIF Reverse primer (10pmol)	1µL
DEPC water	20.5µL
Total volume	50µL

These qPCR master mix reaction components that mentioned in table was placed in sterile white qPCR strip tubes and transferred into Exispin vortex centrifuge for 3minutes, the place in MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as the following table:

qPCR step	**Temperature**	**Time**	**Repeat cycle**
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	10 sec	45
Annealing\ Extension	56.7 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

Results and Dissuasion

The results of clinical examination showed typical lesion ORF disease that lesion developed initially as papules and pustules, than thick tenacious scraps covering a raised area of ulceration, granulation and inflammation. Fig (1-A). There was severe systemic reaction. The lesions occur in the mouth involving the gums, dental pad or combination. There was extensive painful and proliferative lesions occur on gingival margins of incisors teeth and within mouth. Fig (1-B)

Figure (1): Clinical cases showing typical signs of contagious ecthyma (ORF) in lambs A: Papules and Pustules, B: The lesions in the mouth involving the gums and dental pad.

The clinical sign that explain in this study was in agreement with (8, 12) who referred to the typical lesions of ORF virus infection on lips, gums, dental pad, as well as oral mucosa. The results showed that there was high spreading of infection in newly born lamb. It may be due to not well develop the immune status. The results also showed there was sever infection in lamb that suggested the animals infected with highly virulence ORF virus strain.

On the other hand, the results of molecular diagnostic technique, Real-Time PCR were showed specific rapid detection of GIF gene ORF virus out

from (21/23) at percent (91%). The GIF gene ORF virus that amplification by SYBR Green dye based Real-Time PCR. As show in Real-Time PCR amplification, Fig (2), Fig (3). The specifity Real-Time PCR amplification of the positive samples show specific amplification at melt peak mainly at (Tm: 80C°) without primer diamer or nonspecific products. Fig (4)

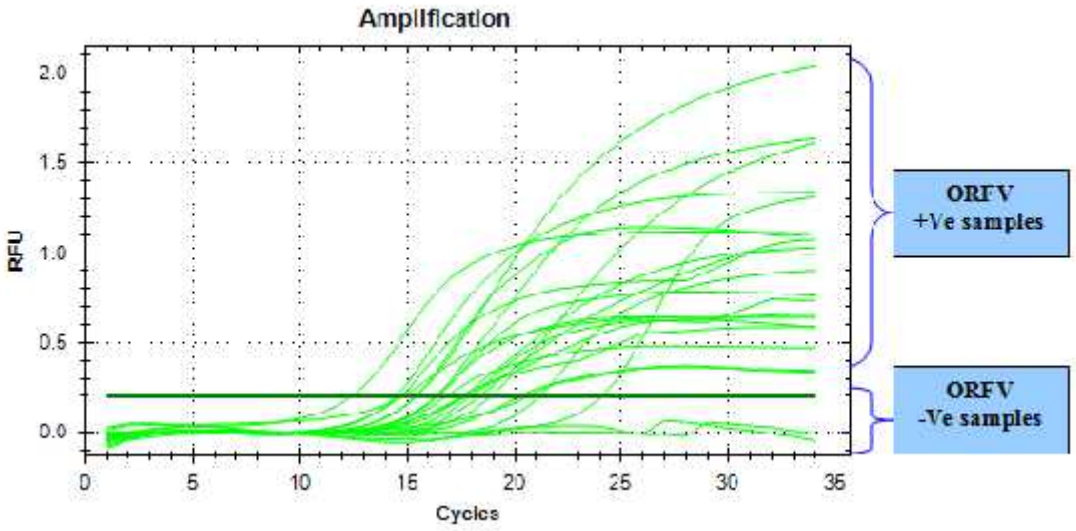

Figure (2): Real-Time PCR amplification plots GIF gene ORF virus in lip scarp tissue samples of lambs.

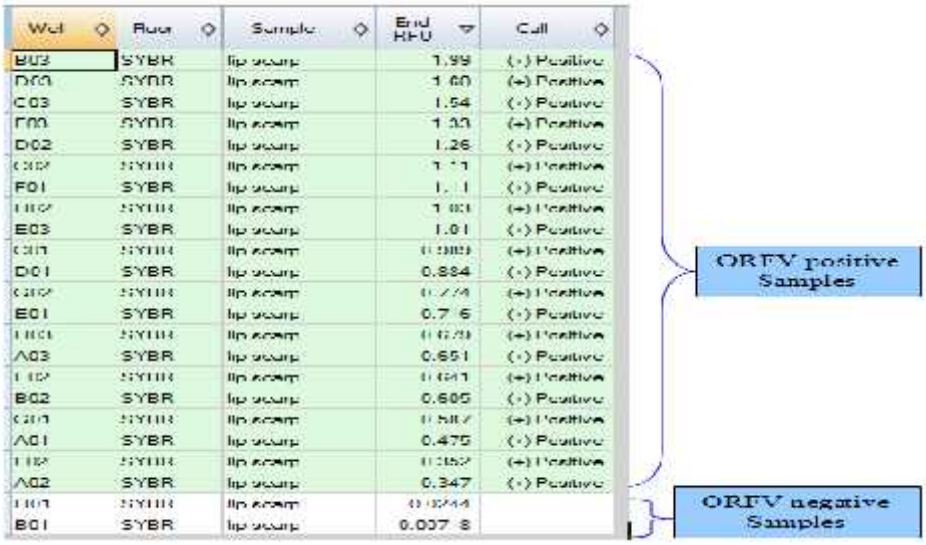

Figure (3): Real-Time PCR end point diagnosis results of GIF gene ORF virus

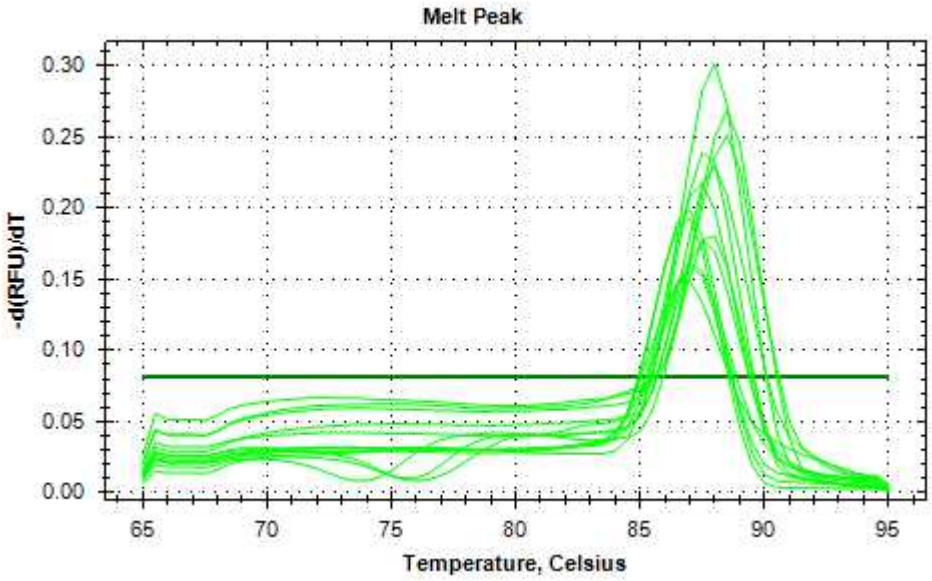

Figure (4): Real-Time PCR Melt curve that shows the melting point for ORF virus GIF gene ranged from 87°C to 88 °C for positive samples.

Real-Time PCR technique that used in this study was appear highly sensitive and rapid tools in specific diagnosis of ORF virus. These results were consistent with (11) who that rapid, sensitive SYBR green-based real-time PCR (RT-PCR) assay could be used as a routine diagnostic tool for bulk processing and analysis of ORFV-suspected clinical samples as it holds better when c++ompared to the conventional PCR that is currently being routinely used for rapid diagnosis in our laboratory. Diagnostic method by electron microscope is rapid and can be distinguishes ORF virus from other parapoxviruses particles; however, electron microscope facilities with experienced personnel are not universally available at present. Even if an electron microscope diagnostic laboratory is available, electron microscope diagnoses require high viral loads of approximately (10^6 particles/mL) with an intact morphology Such a load usually occurs in fresh lesions and crusts

of infected individuals, but in cases of lower viral loads or material not preserved according to electron microscope requirements, electron microscope may produce false-negative results (13).

Convential molecular technique such as PCR is well recognized to be a stable, fast, and sensitive diagnostic method for the detection of nucleic acids, although it cannot confirm the presence of complete or infectious particles (14). However, in order to get an accurate diagnosis of ORF virus in sheep; we therefore developed a real-time PCR-based assay. It is fast, specific, and reliable, with a decreased risk of carryover contamination, and it makes large-scale diagnoses possible.

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التشخيص السريع لداء الحميقاء الساري (فيروس الاورف) في محافظة الديوانية بواسطة تقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي

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الخلاصة

داء الحميقاء الساري هو مرض جلدي فيروسي شديد العدوى، ومن الأمراض المشتركة بين الإنسان والحيوان ، حيث يصيب المرض الأغنام والماعز وبعض الحيوانات المجترة المستأنسة والبرية الأخرى. غالبا ما يكون تشخيص المرض سريرياً ممكنا، ولكن في بعض الأحيان التشخيص المختبري يكون ضرورياً. حيث طرق التشخيص المصلي متوفرة ولكن يبقى المجهر الإلكتروني هو الطريقة الأكثر استخداماً للتشخيص. مع ذلك، بالنظر لعدم توفر المجهر الالكتروني في العديد من مختبرات التشخيص البيطرية وتكلفته العالية. لذا لا بد من أجراء طريقة عملية موثوق بها لتشخيص عدوى فيروس الاورف. إن تطور طريقة فحص تفاعل البلمرة المتسلسل في الوقت الحقيقي للكشف الجزيئي السريع عن الحمض النووي الفيروسي لفيروس الاورف يعتبر أداة تشخيص مختبرية متخصصة وحساسة للكشف عن داء الحميقاء الساري. ولقد تم أجراء فحص تفاعل البلمرة المتسلسل في الوقت الحقيقي من خلال تصميم البادئات المتخصصة لتضخيم منطقة عالية الثبات من جين (GIF) لفيروس الاورف بحجم (١٤٧ زوج قاعدي) ، والتي تكون مسؤولة عن تشفير إنزيم العامل المثبط المقاوم للمناعة. أظهرت نتائج هذه الدراسة تشخيص دقيق وحساس لفيروس الاورف في عينات بثور نسيج الشفاه لصغار الأغنام (٢١ عينة موجبة من ٢٣ عينة) بنسبة (٩١%). نستنتج من هذه الدراسة بان تقنية فحص تفاعل البلمرة المتسلسل في الوقت الحقيقي يوفر طريقة سريعة وحساسة للكشف الدقيق والتشخيص المباشر لداء الحميقاء الساري (فيروس الاورف) في الأغنام.

مفتاح البحث: داء الحميقاء الساري، فيروس الاورف، الأغنام، تفاعل سلسلة البلمرة الآني.