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Research article

Phylogenic analysis of the sheep Orf virus in Iraq

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Abstract

Orf disease is a highly contagious, zoonotic viral skin disease affects sheep, goats and some other domesticated and wild ruminants, therefor this study is designed to evaluate predominant genotypes circulating Orf virus of sheep in Al-Qadisiyah Province in Iraq as the first time by using the sequencing and phylogenic analysis of isolates Orf virus strains. The study was carried out on (94) skin samples of affected sheep animals diagnosed previously by RT-PCR technique in different ages and sexes between September /2013– March/ 2014. PCR assay was performed for amplification of 408bp GM_CSF/IL-2 inhibition factor (GIF) gene Orf virus in positive samples of real-time PCR assay. Eight purified DNA products isolated from different areas of Al-Qadisiyah Province (one sample for each area) were analyzed by using sequencing method to obtain the nucleotide sets of GIF gene. DNA sequencing technique was performed for Phylogenic relationship analysis study of local Orf virus positive samples isolates with NCBI-GenBank Global Orf virus isolates. The results of endpoint polymerase chain reaction for detection of specific GIF gene (408 bp) of Orf virus showed positive results for all collected examined samples. Sequencing analysis of our isolates in the present study recorded and published under accession numbers were (KJ508895.1, KJ634611.1, KJ634842.1, KJ648451.1, KJ653445.1, KJ653446.1, KJ659373.1 and KJ697772.1). The results of phylogeny tree construction showed that five of our published isolates of this study (KJ508895.1, KJ648451.1, KJ653445.1, KJ653446.1, and KJ697772.1) are located in the same first tree branch, which shared highest and closed relationship with AF192803.1, DQ184476.1 Orf virus strain NZ2 isolated from New Zealand. The KJ659373.1 our published Iraqi Orf virus isolates of this study showed high homology and closed relationship with AY605973.1 Orf virus isolated from reindeer in Norway. Phylogenic analysis of KJ634611.1 and KJ634842.1 our published Iraqi Orf isolates of the present study showed high homology with AY605977.1 Orf virus isolated from goat in Norway. Sequencing study and phylogenic analysis have been considered useful in understanding Orf virus scenario in Iraqi endemic area and which important in application of control measures and selective efficient vaccines to use in vaccination programs in Iraq.

Keywords: Orf disease, Sheep, Parapoxvirus, PCR assay, Sequencing.

Introduction

Orf disease is a highly contagious, zoonotic, viral skin disease affects sheep, goats, and some other domesticated and wild ruminants. It is also known as contagious pustular dermatitis (CPD), contagious ecthyma, infectious labial dermatitis and scabby mouth, or sore mouth. (1). The causative agent of the disease is a *parapox* virus (Family *Poxviridae*, Sub-family *Chordopoxvirinae*) (2), it has linear double-

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stranded DNA dermatologic and epitheliotropic virus (138 kbp) (3). Morbidity of the disease can be very high, approaching 100%, but the mortality rate in uncomplicated cases rarely exceeds 1%. In this regard, secondary staphylococcal infection is a frequent occurrence and mortality rates may be from 20% to 50% in contaminated herds. The mortality occurs, especially in young sucking lambs, due to an incidence of dehydration and starvation, as the pain and distortion of the lips and mouth preclude the lamb from sucking (1). Lesions are most commonly localized in and around the mouth and nostrils. The lesions in clinical term, progress through the erythematous macula, papule, vesicle, pustule and scab formation. In uncomplicated contagious ecthyma (CE), natural recovery takes three to six weeks, with shedding of scab materials contaminated with virus. Prolongation of the infection and an increase in severity are nearly associated with secondary bacterial infections (4). Orf virus genome has been shown to encode factors that either mimic host immuno-regulatory proteins (5, 6, 7) or has the potential for interacting with components of the host immune system (8, 9, 10). In doing so, it is thought that these virus-encoded factors are capable of subverting the host immune response to infection, creating an environment suitable for efficient virus replication. Orf virus has been shown to encode a protein inhibitory factor gene (*GIF*) that is capable of binding and inhibiting both of the ovine cytokines interleukin-2 (IL-2) and granulocyte– macrophage colony-stimulating factor (GM-CSF). Such a protein has not been found so far in any other *poxvirus*; investigated whether it was conserved in other PPVs. (11). Function of *GIF* speculated in Orf virus infection only. Ovine granulocyte– macrophage colony-stimulating factor (ovGM-CSF) and ovine cytokines interleukin-2 (IL-2) mRNAs have been detected in skin biopsy specimens obtained during Orf virus reinfection (12). The role of GM- CSF is involved in the activation of neutrophils and macrophages, both of which are present in Orf lesions. Macrophage colony-stimulating factor (M-CSF; CSF-1), granulocyte colony-stimulating factor, and IL-3 are also hematopoietic growth factors that support the development and activation of neutrophils and macrophages. (13). Molecular techniques as both Real-Time PCR and conventional PCR were rapid, specificity and highly sensitive technique to detect of *GIF* gene of the Orf virus. In addition to enhanced sensitivity, the benefits of real-time PCR assays over conventional endpoint detection methods include their large dynamic range, a reduced risk of cross contamination, an ability to be scaled up for high throughput applications and the potential for accurate target quantification. The combined properties of high sensitivity and specificity, low contamination risk, and speed has made real-time PCR technology a highly attractive alternative to tissue culture- or immunoassay-based methods for diagnosing many infectious diseases (14). Sequence analysis of virulent Orf virus from the field outbreaks gives an idea about the circulating virulent Orf virus and its comparison with Orf vaccine virus. The other Orf virus sequences in the GenBank will throw light on the molecular epidemiology and emergence of any new variant Orf virus in the field. So the sequence study is considered useful in understanding Orf virus scenario in the endemic areas. (15).

Material and Method

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 434

This study was carried out between September / 2013– March / 2014 by examination of (17) Sheep Flocks from different regions in Al-Qadisiyah Province,

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Summer, Daghara, Afak, Shinaffiea, Sadder, Muhanawia, Shaffiea and Sannia, total numbers of these animals were (4070) animals in different age and sex showed skin lesions such as papules, blisters, pustules and scab. Ninety- six (96 samples) from different regions (12 samples of skin scabs (lip scrape) for each region) from different age and sex animals were collected by using sterile artery forces and thumb which cleaned by tooth brush and dipping in Ethyl Alcohol 96% in closed beaker for 5 minutes, then dried by sterile cotton every time after each collection. Each scab sample was collected in 50ml sterile continuer. The samples were transported as soon as possible to the laboratory by cooled box. 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. Ninety– four (94) positive purified DNA samples with Real-Time PCR master mix reaction used for PCR master mix preparation, PCR assay was carried out by using forward primer (GCTCTAGGAAAGATGGCGTG) and Reverse primer (GTACTCCTGGCTGAAGAGCG) that amplify, (408bp) PCR product were provided by GenBank of *Orf virus* strain *GIF gene*, complete sequence NCBI Reference Sequence: *AF192803.1* (10). PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume by added 5µl of purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer, then the PCR premix tube was completed by deionizer PCR water into 20µl and briefly mixed by Exispin vortex (Bioneer. Korea). The reaction was performed in a thermo cycler (MyGene. Korea) by set up the following thermo cycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 56 °C for 30 s, and extension 72 °C for 45 s and then final extension at 72 °C for 7 min. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination. PCR product was purified from agarose gel by using (EZ EZ-10Spin Column DNA Gel Extraction Kit, Biobasic). Specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL microcentrifuge tube. 400 µl Binding Buffer II was added to gel fragment. After that, incubated at 60ºC for 10 minutes and shacked until the agarose gel was completely dissolved. Above mixture was added to the EZ-10 column and let stand for 2 minutes and Centrifuged at 10,000 rpm for 2 minutes and flow-through in the tube was discarded. After that, 750 µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Solution was discarded. After that, the step 4 was repeated. Then, centrifuged at 10000 rpm for an additional minute to remove any residual wash Buffer. After that, the column was placed in a clean 1.5ml microcentrifuge tube and 30µl of Elution Buffer was added to the center of the column and was incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and stored at -20ºC. The PCR products sequenced in both directs by using (ABI prism DNA sequencing kit) and an (ABI sequencer Bio system). DNA sequencing Analysis:

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Nucleotides sets of different isolates from different regions of Al-Qadisiyah Province were confirmed with others world strains by using NCBI- Blast of nucleotides sets and analysis of these nucleotides sets. Then submitted nucleotides sets of different regions of Orf virus isolate directly through NCBI- GenBank in program Bank It and the nucleotide sequence data in this paper recorded and published in the GenBank, nucleotide sequence databases of *Orf virus*, partial sequence based on *GIF* gene with the following accession numbers: (KJ508895.1, KJ634611.1, KJ634842.1, KJ648451.1, KJ653445.1, KJ653446.1, KJ659373.1 and KJ697772.1) for (Summer, Daghara, Afak, Shinaffiea, Sadder, Muhanawia, Shaffiea, Sannia) respectively. Phylogenic molecular analyses: Nucleotide sets were prepared for identity score and alignment between isolates in this study and other world strains by using NCBI-Clustal W2 program online. Then all world strains identity with Iraq isolates range between (95% - 98%) in identity score was analysis to building phylogenic tree by using neighbor method to designed tree with the MEGA 4 software (16). Phylogenic trees were inferred with distance, parsimony and maximum likelihood methods (19), and the reliability of the trees were determined by 1000 data set bootstrap resembling and cutoff was 50%. (20).

Results

The results of endpoint PCR for detection *GIF* gene of Orf virus in ethidium bromide – stained agarose gel using specific primers for detection *GIF* (408 bp) and the ladder in size was (10000 bp), 12 tissue samples isolated from different regions gave positive results ,as shown in Figure (1). In addition to confirmative diagnosis of the virus, the PCR products used for collection of the target DNA and purification until used in the sequencing method for analysis of *GIF* gene of predominate strain of Orf virus in Iraq.

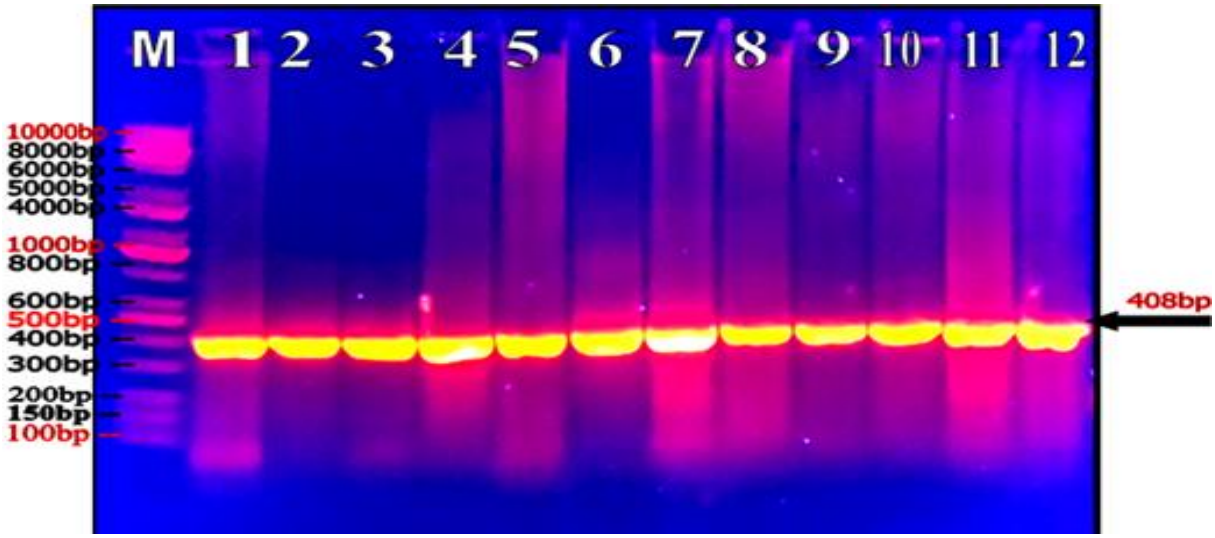

Figure (1): Results of positive Orf samples GIF gene (408bp) by using PCR technique

The results of previous samples of GIF gene analyzed and took the accession numbers as *(KJ508895.1, KJ634611.1, KJ634842.1, KJ648451.1, KJ653445.1, KJ653446.1, KJ659373.1 and KJ697772.1).* These results recorded and published in the International Nucleotide Sequence Database Collaboration (INSDC) this location containing National Center for Biotechnology Information (NCBI), European Nucleotide Archive (ENA) and DNA Bank of Japan (DDBJ). The results of

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the multiple sequence alignment is an important step for phylogenic analysis, which aims at modeling the substitutions that have occurred over evolution and derive the evolutionary relationships between sequences. In addition, the result of multiple sequence alignment can be obtained easily by using (ClustalW2) program, which is a tool to align three or more sequences together in a computationally efficient manner (17). In this study the result of multiple sequences alignment of the partial DNA sequences of (8) Orf Iraqi isolates of the *GIF* (408 bp) gene alignment with others selected reference world Orf DNA sequencing of the same gene of this study showed high identity and similarity . These results primary depending on the BLAST – query nucleotides to detect the percentage of the identity which was ranged from (95-98%) of all selected reference world Orf strains compared with isolates of this study. Phylogenic analysis was performed partially on (408 bp) of *GIF* gene 42 *Parapoxvirus* species strains, including the eight Iraqi Orf isolates of the present study. This analysis revealed that the maximum homology of the nucleotide sequences between the Iraqi Orf isolates and world strain ranged from 94% - 98% nt Table (1). Iraqi Orf isolates showed more homologous and similarity with the only Orf virus strains isolated in the world .Table (1).The results of the phylogeny tree and sequences analysis of the *GIF* coding gene of *Parapoxvirus* revealed that the KJ653446.1 Orf Iraq isolated from Muhanawaia region in Al-Qadisiyah Province Clustaled more together with following strains AF192803.1 Orf virus strain NZ2 isolated from New Zealand, DQ184476.1 Orf virus strain NZ2 isolated from New Zealand, AY386263.1 Orf virus strain OV-IA82 isolated from nasal secretions of a lamb at the Iowa ram test station during an outbreak in 1982 in USA and AY236151.1 Orf virus strain Orf -11 isolated in Norway . The identity score of nucleotides of KJ653446.1 Orf Iraq isolate with clustered strains were (96% nt) of all strains respectively Table (1), It shared highest homology with the AF192803.1 and DQ184476.1 Orf virus, both isolated from New Zealand Figure (5). The Phylogenic analysis and phylogeny tree construction of four our Iraqi Orf isolates of this study (KJ653445.1 , KJ 508895.1 , KJ 648451.1 and KJ 697772.1) from Sadder , Summer , Shinaffiea and Sania respectively located in the same group of phylogeny tree Figure(5) . All these isolates were identical in nucleotide sequences together and occurred in the same tree branch. The results showed this group of our Iraqi Orf isolates recorded in this study were more clustered with selected world reference strains AF192803.1, DQ184476.1, AY386263.1 and AY236151.1 occurred in the first group of first tree branch, Figure (5). The results of identity of (nt) sequences percentage our Iraqi Orf isolate KJ653445.1 of the present study as compared with selected world reference strains AF192803.1 , DQ184476.1 , AY386263.1 and AY236151.1 were (97%,97%,96%and 96% nt) respectively .Table (1). The results of identity score of other Iraqi isolate of the present study KJ508895.1 Orf Iraqi isolate as compared with selected world reference Orf strains AF192803.1, DQ184476.1, AY386263.1 and AY236151.1 were ranged from (98%, 98%,97% and 97% nt).Percentage identity of the KJ 648451.1 Orf Iraq as compared with selected world reference Orf strains AF192803.1, DQ184476.1, AY386263.1 and AY236151.1 were ranged from (98%,98%,97% and 96 nt). Nucleotide and sequences identity percentage of the KJ 697772.1 Orf Iraqi isolate as compared with selected world reference Orf strains AF192803.1 , DQ184476.1 , AY386263.1 and AY236151.1 were ranged from (97%,97%,98% and 96% nt)Table (1). All our four Iraqi Orf isolates shared the highest and closed relationship with AF192803.1, DQ184476.1 Orf virus strain NZ2 isolated from New Zealand when constructed tree using maximum likelihood method and

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pseudo-replicate 1000 as tested of the phylogeny tree. Figure (5). Results of phylogeny tree of KJ 659373.1 our Orf Iraqi isolates isolated from Shaffiea region showed highest homology and closed relationship with the selected world reference Orf strain AY 605973.1 Orf virus isolated from reindeer in Norway Figure (5). Nucleotide identity percentage were ranged from (97% nt). Table (1).

Table (1): Compute identity score % of nucleotide sequences of (*GIF*) gene between Iraqi Orf isolates and some other selected reference world Orf strains.

No	Accession Numbers	1*	2*	3*	4*	5*	6*	7*	8*
1	DQ184476.1	98	98	97	98	97	96	97	97
2	AF192803.1	98	98	97	98	97	96	97	97
3	AY605988.1	98	98	97	98	97	96	97	97
4	AY605971.1	97	96	97	97	97	94	95	96
5	AY605972.1	97	97	97	97	97	97	98	98
6	JF773684.1	98	97	97	97	97	96	97	97
7	JF773685.1	98	97	97	97	97	96	97	97
8	AY605979.1	98	97	97	97	97	96	97	97
9	AY605985.1	97	97	97	97	97	96	97	98
10	AY605973.1	96	96	97	96	96	95	97	97
11	KF837136.1	98	98	97	98	97	96	96	96
12	AY236151.1	97	97	96	97	96	96	97	98
13	AY605986.1	97	97	96	97	96	95	96	96
14	AY605992.1	97	97	96	97	96	96	97	98
15	AY605978.1	97	97	96	97	96	96	96	97
16	AY605974.1	97	97	96	97	96	96	96	97
17	KJ610835.1	96	95	96	96	95	94	95	95
18	KF666567.1	96	95	96	96	95	94	96	96
19	AY605975.1	97	97	96	97	96	95	97	97
20	JF773690.1	97	97	96	96	96	96	97	97
21	JF773691.1	97	97	96	96	96	96	97	97
22	JF773686.1	97	97	96	96	96	96	97	97
23	JF773687.1	97	97	96	96	96	96	97	97
24	HM133903.1	97	96	96	97	96	95	96	96
25	AY386263.1	97	97	96	96	96	96	96	96
26	AY605990.1	97	96	96	97	96	95	96	96
27	AY605976.1	97	96	96	97	96	96	97	97
28	AY605977.1	96	96	96	96	96	95	96	96
29	DQ922634.1	96	96	96	96	95	95	96	96
30	KF726847.1	95	95	95	95	94	93	95	95
31	AY386264.1	96	96	95	96	95	94	95	95
32	KJ610834.1	96	95	95	96	95	94	95	95
33	KF666566.1	95	95	95	95	95	94	95	95
34	AY605982.1	94	94	94	95	93	94	94	94

(1*)KJ508895.1 Orf Iraq, (2*)KJ634611.1 Orf Iraq, (3*) KJ634842.1 Orf Iraq, (4*) KJ648451.1 Orf Iraq, (5*) KJ653445.1 Orf Iraq, (6*)KJ653446.1 Orf Iraq, (7*) KJ659373.1 Orf Iraq, (8*) KJ697772.1 Orf Iraq.

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The phylogeny tree of KJ 634842.1 Orf Iraq isolated from Afak region was highest homology with as compared with selected world reference Orf strain AY605977.1 Orf strain isolated from goat in Norway Figure(5). Nucleotides identity percentage were ranged from (96%) Table (1). The results of phylogeny tree of KJ634611.1 Iraqi Orf virus isolated from Daghara region clustered with AY605977.1 Orf Norway and AY605974 Orf strain isolated from musk ox in Norway .Figure (5). This strain showed highest homology (96% nt) with AY 605977.1 Orf Norway isolated from goat.

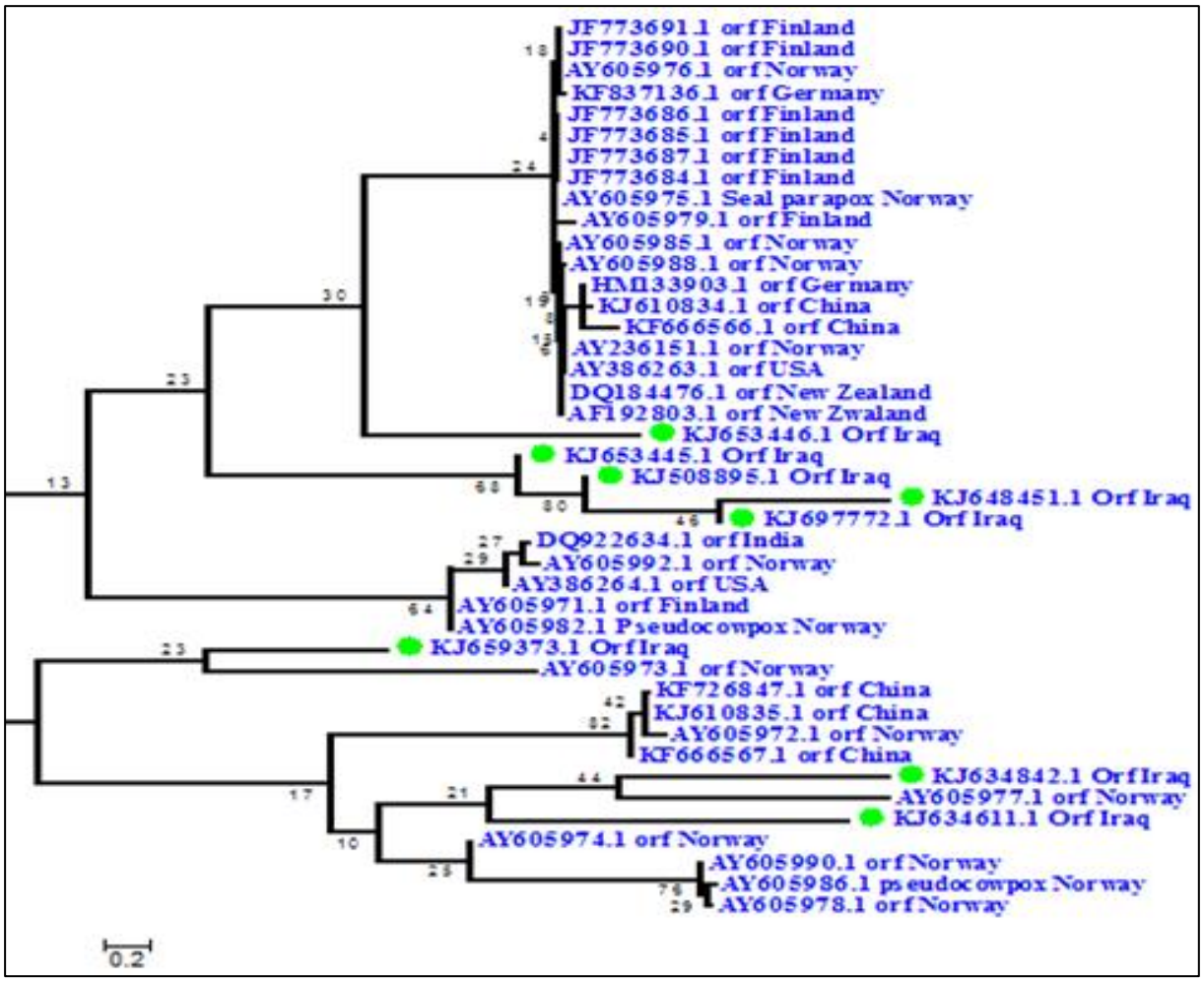

Figure (5): Traditional (Rectangular) phylogeny tree using a neighbor– joining method constructed based on (408 bp) *GIF* gene of Orf virus.
***(Numbers on the tree) units of the number of base substitution per site.**
***Green spots (Iraq Orf isolates).**

Discussion

Orf virus is prototype of *Parapoxvirus* genus , it can cause contagious ecthyma disease in sheep , goats and other worldwide animals .The virus is sometimes transmissible to humans due to direct contact .The viral genome consist of linear double stranded DNA , essential genes are located in the conserved central part of the genome and variability is observed in the terminal ends , genes in near terminal regions of the genome encoded factors with important role in viral – host interactions such as modulating host

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responses to infection and determining host range . The *GIF* gene encode GM_CSF/IL2 protein. The *GIF* gene has been used for detecting of Orf virus by molecular techniques. The present study was conducted to study Orf disease in Al-Qadisiyah Province in sheep by sequencing of GIF gene. Phylogenic analysis of our recorded isolates was shared high homology to New Zealand and Norwegian strains. This is the first study on molecular phylogenic characterization of the Orf virus in Iraq. The PCR results showed high sensitivity in detection of *GIF* (408bp) gene of the Orf virus by using specific primers. These results of PCR were Similar to the results of many scientists who used the conventional PCR for detection of Orf virus by amplification part of *GIF* gene with an amplicon size of 408 bp for detection of Orf virus in tissue samples and for virus strains differentiation. (10, 21, 22, 15).The PCR assay was sensitive enough to detect a minimum DNA concentration of 5 ng total genomic DNA of Orf virus from suspected scab samples. Since the target selection, primer designed and assayed optimization was perfect, this PCR assay assured reproducibility and specificity of the results. Hence, this PCR assay using *GIF* inhibition factor of GM_CSF / IL-2 gene primers system for the diagnosis of contagious ecthyma in the field outbreaks without using cell culture system or electron microscopy as time consuming and high coast of these diagnostic technique. However, in combination with typical clinical pictures, detection of the respective viral nucleic acid must be considered as accurate evidence of infection .The positive PCR products were purified and sent frozenly to the sequence analysis to ensure its relation to ovine *Parapoxvirus* genus. The sequencing study is considered a useful molecular approach in understanding the infectious/contagious virus scenario worldwide. Sequence analysis of virulent Orf virus and its comparison with other Orf virus sequences in GenBank gave an idea on the molecular epidemiology and emergence of any new variant Orf virus in the field , on the other hand it is very important in country measurement of control of the disease Results of GenBank submissions and recording strains of this study helped in the phylogenic analysis by using only accession numbers in phylogenic analyses steps First step of analyses by alignment of all isolates of this study with other world selected reference Orf strains by using online (Clustal W2) program. This program demonstrated had accurate degree of the identity with all world strains including our isolates of this study. These results with (Clustal W2) were important because they used directly in the phylogeny tree construction .The results of phylogeny tree and sequences analysis by using MEGA4 of our isolates under accession numbers KJ653446.1, KJ653445.1, KJ508895.1 , KJ648451.1 and KJ 697772.1 showed the highest homology with other world strains as AF192803.1 and DQ184476.1 Orf strain isolated from New Zealand. KJ659373.1 showed the highest homology with the AY605973.1 Orf virus isolated from reindeer in the Norway. Our isolate KJ634611.1 and KJ634842.1 showed highest homology with AY605977.1 Orf Norway isolated from goat. All results of the present study showed relationships between the geographical locations of the Orf recorded strains of this study and results of the phylogeny tree with suspected source of the introduction and spread of the disease in Al-Qadisiyah Province. Such relationships when they are analyzed according to the geographical location of the five strains of this study KJ653446.1, KJ653445.1, KJ648451.1, KJ 697772.1 and KJ659373.1 Orf Iraq strains were isolated from Muhanawaia , Sadder, Shinaffiea, Sania and Shaffiea regions respectively that located in the west south , west north , and west of the Al-Qadisiyah. The affected flocks in Al-Sadder town were exactly located in the west of the town between Al- Sadder and Al-Shaffiea. In addition, the Al Sania that

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located in north of city but the flocks which infected with Orf outbreaks located in west side between Sania and Al Muhanawaia. Furthermore, all databases about affected flocks in these regions demonstrated the owners were newly came to the Al-Qadisiyah City from grazing area of the western area of Iraq. KJ634611.1 and KJ634842.1 our Orf Iraq recorded strains from Daghara and Afak regions respectively, these regions located in the east north and east of Al-Qadisiyah Province. The sudden outbreaks occurrence recorded in Afak region in (Jalehha area) that located in the east south of Al-Qadisiyah. Also the flocks from AL-Daghara located in east of this region near Al- Sania, also some flocks recorded outbreaks of the disease in Summer which located between Afak and Al-Daghara regions near Al-Shomali region in west of Al-Qadisiyah Province. These results indicated the disease spread primary in the east and southeast Al-Qadisiyah and spread to other regions. Also these results indicated the disease enter and spread from outer boundary to Al-Qadisiyah during movement of the effected animals in western Iraq during summer season to the middle and south Iraq and rapid spread of infection in the selling animals stations inside the cities. These results may confirm disease spread from Al Saudi -Arabia to the Iraq due to the infection of the livestock of the Al Saudi-Arabia with effected sheep during importation of animals during the period of pilgrims, especially from New Zealand. The results that showed the highest homology with the Norwegian strains may be related to the use of different vaccine from different countries in sheep and goat in Al Saudi Arabia to reduce outbreaks and control the disease.

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