Phylogenetic analysis of B2L gene of Egyptian orf virus from naturally infected sheep

Mohamed El-Tholoth¹ · Yasser F. Elnaker² · Gamal Shiha³

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Abstract Orf is a viral disease caused by a parapoxvirus, affecting primarily sheep and goats and causes severe economic losses. In this study, a total of 500 sheep from a farm in El-Beheira Governorate, Egypt were examined during spring, 2014. Out of them, 30 sheep showed clinical signs of orf virus infection. The diseased sheep exhibited proliferative lesions on the lips and around the mouth. Polymerase chain reaction (PCR) was used for diagnosis of the disease. For genetic characterization of the Egyptian orf virus, the sequence of a major and highly immunogenic envelope protein gene (B2L gene) was identified and compared with the sequences available from different parts of the world. The virus was detected in 24 out of 30 collected samples (80 %) by PCR. Phylogenetic analyses of the Egyptian orf virus B2L gene showed close genetic relationship with Israel orf viruses those were identified in 2012. In conclusion, this study reports identification and genetic characterization of Egyptian orf virus in sheep in Egypt.

Keywords B2L gene · Egypt · Orf virus · Polymerase chain reaction · Sequencing analysis · Sheep

Introduction

Orf, contagious pustular dermatitis (CPD), contagious ecthyma (CE) or ovine pustular dermatitis (OPD) is a common viral skin disease of sheep and goats caused by a parapoxvirus [6]. Parapoxviruses are distinguished from other poxviruses by the ovoid virion shape, the criss-cross pattern on the particle surface, and the relatively small size and high G+C content of the genome (~140 Kb) [3]. The virus B2L gene (~1137 bp) encodes a major and highly immunogenic envelope protein which is a homologue of vaccinia virus major envelope antigen p37K [14].

The disease usually occurs in young sheep and goats (3–6 months old) but neonatal lambs and kids aged 10–12 days old are highly susceptible and showed severe clinical signs [12]. The virus is an epitheliotropic virus that causes proliferative lesions in the lips, around the nostrils, oral mucosa and sometimes also affects the gums and tongue [6]. The disease runs for 3–4 weeks and usually resolves in 2 months but mortality rates up to 10 and 93 % have been reported in lambs and kids, respectively [11].

The disease causes severe economic losses due to young animals losing condition, slower growth rate, trading restrictions and death of lambs. The virus can survive on the premises in scabs from lesions, for up to 15 years [13]. Several outbreaks of the disease have been reported in Egypt [1, 10].

Diagnosis of orf virus infection is based on clinical signs, virus isolation, electron microscopy, histopathology and serological tests [1, 2, 5, 8]. These conventional diagnostic methods are time and labor consuming.

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✉ Mohamed El-Tholoth
tholothvirol@mans.edu.eg

¹ Department of Virology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt

² Department of Animal Medicine (Infectious Diseases), Faculty of Veterinary Medicine, New Valley Branch, Assiut University, Asyût, Egypt

³ Veterinary Medicine Directorate-El Beheira, Ministry of Agriculture, Damanhour, Egypt

Polymerase chain reaction (PCR) has been described for the detection of orf virus and proved to be rapid and specific diagnostic method [1, 2, 7, 9]. Rapid diagnosis of the disease followed by rapid implementation of control measures is very important to control the transboundary transmission of the disease.

The aim of this study was to identify orf virus in diseased sheep showed clinical signs suspected to be infected with the virus in El-Beheira Governorate, Egypt during spring, 2014 using PCR as well as phylogenetic characterization of the partial sequence of the B2L gene.

Materials and methods

Animals and samples

During March, 2014, nodular lesions appeared on the lips and around the mouth in sheep farm in Noubaria, El-Beheira Governorate, Egypt. A total of 500 sheep were examined. Out of them, 30 sheep showed clinical signs suspected to be orf disease. Clinical examination of these animals revealed mild fever (up to 41 °C), nodular lesions confined to the skin around the mouth (Fig. 1), the inner side of lips and gums and Pneumonia. The affected sheep were aged between 1 month old and 6 months old. Sheep older than 6 months did not exhibit any clinical manifestation. The veterinarians treated the affected sheep with oxytetracyclin at the dose rate of 10 mg/kg B.Wt. per day for 4 days to avoid bacterial infections. Betadine dressing was applied to the lesions to prevent secondary infections. Intramuscular injection of 1 % dextrose saline and multi-

Fig. 1 Typical case of orf virus infection in sheep with proliferative lesions around the mouth

vitamins was used as a supportive treatment. After about 2 weeks, the animals recovered from the clinical signs.

Skin lesions were collected from all clinically affected animals. The sample was taken in dry bottle containing phosphate buffer saline (PBS) for PCR identification of orf virus. Skin biopsies from two normal sheep were included as negative controls.

Preparation of skin biopsies for virus identification

Skin lesions were minced using sterile scissors and forceps and then homogenized in a mortar containing sterile sand with a pestle. Phosphate buffered saline was added, making a 20 % (W/V) suspension. The suspension was centrifuged at 720×g for 10 min. The supernatant was collected and stored at −20 °C till used.

Polymerase chain reaction (PCR)

PCR was used to identify orf virus from skin lesions of diseased sheep. Oligonucleotide primers were designed by [7] and synthesized by Metabion International AG Company, Germany. The primers were received in lyophilized form and resuspended in Tris/EDTA (TE) buffer to reach a final concentration of 100 pmol/μL and were designed to amplify a specific segment of B2L gene of orf virus (594 bp). The primers sequences for PCR amplification were as follows: forward primer (PPP-1), 5'-GTC GTC CAC GAT GAG CAG CT-3' and reverse primer (PPP-4), 5'-TAC GTG GGA AGC GCC TCG CT-3'.

DNA extraction was performed as published previously [15] using 0.5 mL of the prepared skin lesion suspension which was digested with 20 μL Proteinase K (final concentration, 100 μg/mL) at 56 °C for 2 h. 100 μL Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed by inversion then centrifuged at 17,950×g for 5 min then the supernatant was transferred to a clean microcentrifuge tube and 2.5 volumes absolute ethanol and 1/10 volume of 5 mol/L sodium acetate (pH 5.3) were added and mixed thoroughly. The DNA was precipitated at −20 °C overnight and pelleted by centrifugation at high speed (17,950×g) for 15 min. The pellet was washed once with 70 % ethanol and centrifuged at 17,950×g for 10 min then air dried and resuspended in 50 μL TE buffer. Negative control samples were included. Amplification of extracted DNA by PCR was carried out as described previously [7]. Briefly, 10 μL sample of extracted DNA of each isolate was placed in 50 μL of the final volume of 10 × reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 200 mmol/L of each dNTP, 100 pmol of each oligonucleotide primer and 2 U Taq-DNA polymerase. Then 40 μL of mineral oil was added to prevent evaporation of

components during thermocycling. The PCR had an initial cycle of 95 °C for 9 min, followed by 25 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final elongation step of 72 °C for 7 min. Amplified product analysis by agarose gel electrophoresis was carried out according to previous report [15]. Briefly 10 µL of the amplified PCR product was mixed with 1 µL 10× gel loading buffer and loaded to the individual wells of a 1.5 % agarose gel. In addition, 2 µL of a 100 bp DNA molecular weight marker was loaded with 2 µL loading buffer be used as DNA ladder. The amplified DNA products were detected in comparison with DNA ladder using the U.V. transilluminator.

Nucleotide sequencing and analysis of sequencing data of PCR product

PCR amplified products were gel purified using DNA gel extraction kit (MONTAGE, MILLIPORE) and sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit on a 3130 sequencer (Applied Biosystems). The obtained sequences data were analyzed using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and then the alignment *.aln output file was used for performing the neighbor-joining (N-J) phylogenetic analysis and calculation of divergence and identity percents were done using MegAlign (DNASTAR, Lasergene®, Madison, WI).

Results

Clinical examination

Clinical examination of a farm containing (500) sheep in El-Beheira Governorate, Egypt revealed that a total of 30 sheep exhibited clinical signs suspected to be orf disease in form of proliferative lesions appeared on the lips and around the mouth. The young age's sheep (1 up to 6 month) were affected, while the ages older than 6 months did not exhibit any clinical manifestation.

Identification of orf virus in collected skin lesions by PCR

Analysis of the PCR products obtained from the amplification reaction of extracted DNA from skin lesions of diseased sheep by agarose gel electrophoresis revealed the positive amplification of the B2L gene of orf virus with correct size (594 bp) in 24 out of 30 collected samples (80 %). None of the control negative samples showed a positive reaction. This means that incidence of the disease was 4.8 % (24 out of 500).

Nucleotide sequencing and analysis of sequencing data

After PCR identification, sequencing of the partial amplified B2L gene was done. The obtained nucleotide sequence was submitted to GenBank (accession no. KP984529) Comparing our sequence with those B2L gene sequences available on GenBank (Supplementary Table 1) from different countries (Israel, Korea, Brazil, Greece, Turkey, Taiwan, Japan, China, Finland and India) was carried out. The multiple alignments revealed that there is no significant difference among the different orf viruses from different parts of the world based on the viral B2L gene sequences. The phylogenetic analysis (Fig. 2) revealed that our isolate is genetically closely related to Israel virus (KF985229). Our Egyptian orf virus showed to have 99.2 %, 97.5 and 97.7 % identity with Israel viruses with accession number KF985229, KF985227 and KF985226, respectively while shared an average identity of 90.5 % with turkey and Taiwan orf viruses with accession numbers JQ936990 and EU327506 (Supplementary Table 2).

Discussion

Orf is a highly contagious viral disease of sheep, goat and occasionally human with a world wide distribution caused by a virus in the *Parapoxvirus* genus, subfamily *Chordopoxvirinae* within the family *Poxviridae* [6]. Young

Fig. 2 The phylogenetic tree based on B2L gene nucleotide sequences of our orf virus from sheep with others orf viruses whose B2L genes were from the GenBank database. Numbers at the internal nodes represent the bootstrap probabilities (1000 replicates)

animals as well as adults may be affected. Vaccination has a limited effect and just decreases the severity and duration of the disease. Also, vaccination of flocks free of the disease is not recommended [13]. There is no effective vaccine to control the disease in Egypt [10]. The disease has the ability to cause severe economic losses in the form of decrease in productivity, negative effect on animal welfare and mortality rate ranges between 5–15 % [1, 10].

In this study, we describe occurrence of orf disease in sheep farm in El-Beheira Governorate, Egypt. The causative virus, orf virus was identified by PCR and genetic characterization of the Egyptian virus depending on B2L gene was done. Low incidence of the disease in this study (4.8 %) may be attributable to sample size and sampling place. This study confirmed infection of sheep in El-Beheira Governorate, Egypt with orf virus. The used treatment regime was effective in recovering the animals. This treatment regime is similar to that reported previously [9].

Further studies on risk factors for this infection are necessary especially in developing countries like Egypt where sheep are raised with varied management.

The study confirmed that PCR could be used as rapid, sensitive and economic method for laboratory confirmation of orf virus infection that reduces the dependence on tissue culture and/or chorioallantoic membranes (CAMs) of embryonated chicken eggs (ECEs) and the time required to isolate the virus. Although laboratory diagnosis of the disease could be achieved by electron microscopy, virus neutralization and enzyme linked immunosorbent assay (ELISA) but these techniques have some drawbacks. For example, electron microscopy is expensive and its equipments are not available in most of laboratories in developing countries. Virus neutralization is time and labor consuming and ELISA is not available commercially in most of affected countries.

Genetic characterization of Egyptian isolates have been reported by previous studies [1, 10] but none of these studies made genetic characterization depending on B2L gene that considered a major and highly immunogenic envelope protein [14]. Also, no previous studies compared our Egyptian orf virus with Israel viruses. Sequence analysis of partial sequence of the B2L gene of Egyptian orf virus showed close genetic relationship with Israel viruses that identified in 2012. This indicates that the virus is spreading between Egypt and neighboring countries. Further study is required to identify the origin of this virus and analysis of virus spread pattern. Also, Future work should be directed toward phylogenetic study based on late transcription factor (VLTF-1) gene of orf viruses that sequenced in previous studies in Egypt [1, 10].

There is a high degree of nucleotides homology among all orf viruses for different parts of the world and this is

may be due to the DNA polymerase proof reading activity [4].

In conclusion, findings of this study reveal that orf virus is present in sheep population in El-Beheira Governorate, Egypt during 2014. This study reports genetic characterization of B2L gene of Egyptian orf virus and revealed that Egyptian orf virus has a close genetic relationship with Israel orf viruses.

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