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Isolation and Molecular Diagnosis of Orf Virus from Small Ruminants and Human in Egypt

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Abstract

ORF virus of sheep and goats is one of several zoonotic parapoxviruses. Molecular and serological diagnosis of ORF virus provides high sensitivity methods for accurate and rapid diagnosis for Orf virus infection in sheep, goat and human in Egypt. The present work aimed to isolate and characterized of Orf virus isolated from sheep, goat and human and determined the efficacy of Negilla Sativa antiviral activity. All biopsy samples from human and animals were prepared and inoculated on chorioallantoic membranes of embryonated chicken eggs for virus isolation. The isolated virus was identified and characterized by Enzyme linked immune sorbent assay, Fluorescent antibody technique, electron microscopy and polymerase chain reaction. The isolated virus give specific green fluorescence, Micrograph showed ovoid shape particles 290-300×160 nm in diameter and PCR product (B2L gene) fragments approximately 592 bp which similar to reference Orf virus. The positive Orf virus antibodies in the serum samples by protein A ELISA, positive samples were 4 out 3, 9 out 29 and 18 out 48. Also, by IFAT were 3 out 39, 6 out 29 and 12 out 48 and by AGPT were 1 out 39, 5 out 29 and 7 out 48 in human, goat and sheep at Beni-suef Governorate, Egypt respectively. The ORF virus treated with Negilla Sativa essential oil effect on Orf virus, it reduced the virus infectivity titer from 6.9 Log_{10} to 1.5 Log_{10} by EID 50/0.2 ml. The Orf virus sensitive to the effect of temperature at 37°C and 56°C/6hr were showed reduction in the virus titer with variable Degrees. It was concluded that the PCR and protein A ELISA proved to be more rapid, simple and sensitive for detection of ORF virus infection in human and animals, Negilla Sativa essential oil has antiviral effect against Orf virus but still need extensive research for chemical composition analysis to detect active principle.

Keywords: Molecular; Contagious ecthyma; ORFV; ELISA; FAT; PCR

Introduction

The family of Poxviridae consists of large, enveloped DNA viruses that are of veterinary and medical importance [1,2]. Members affecting wildlife include parapoxvirus, buffalo-pox virus, squirrel parapoxvirus, monkey-pox virus, dolphin poxvirus and the poxvirus that causes myxomatosis in rabbits [3]. Parapoxviruses cause contagious pustular dermatitis or orf in sheep and goats, papular stomatitis and pseudocowpox in cattle [4]. Contagious ecthyma is an acute, contagious, debilitating and economically important zoonotic viral skin disease that affecting sheep, goat and some other domesticated and wild ruminants [5]. Contagious ecthyma is a nonsystematic eruptive skin disease worldwide distribution [3,6]. Also, known as sore mouth, Contagious Pustular Dermatitis (CPD), scabby mouth and usually more severe in goats than in sheep [7-10]. Contagious ecthyma is manifested by proliferative lesions on the mouth and muzzle [11,12], as well as humans [13]. Orf virus represent an occupational health hazard for farmers, abattoir workers, veterinarians, and sheep shearers who handle sheep and goat infected with Orf virus by direct contact or indirect contact with slaughter sheep and goat hide or contaminated objects [11,14], skin nodules is typically found on the human hands and finger [15,16].

Orf virus has 134–139-kb linear double-stranded (DNA) genome [1]. Virion shape and size is ovoid shape have 290-300 nm in length and 160 nm in width diameter outer membrane of a single long spiral tubule wrapped around a homogenous core [17].

In Egypt, Orf virus was firstly observed among an imported flock of foreign breed sheep [18], then several outbreaks of variable severity were recorded [19]. Laboratory diagnosis of the disease is achieved by negative stain electron microscopy from scabs of affected animals [20]. Many serological tests used for diagnosis of Orf virus include Fluorescent Antibody Technique (IFAT), Virus Neutralization Test (VNT), Agar Gel Immunodiffusion (AGID) and Enzyme Linked Immunsrbant Assay (ELISA) [21-23]. The development of Polymerase Chain Reaction (PCR) methods for the molecular detection of DNA has met the demands for specific and sensitive laboratory diagnosis of the disease [24-27]. The presented work aimed to characterize of Orf virus isolated from sheep, goat and human and determined the efficacy of Negilla Sativa antiviral activity.

Materials and Methods

Sample collection:

This method was performed according the guidance of [4,10]. An outbreak during July and August 2013, severe proliferative dermatitis, lesions eventually develop into thick, brown, rapidly growing scabs over areas of granulation, inflammation and ulceration (papules, pustules and vesicles) on the lips, nose, ears and eyelids in young sheep and goat flocks at El Wasta, Nasar and Beba cities in Beni Suef

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Governorate, Egypt. Thirty five samples were collected on 50% glycerin buffer saline from affected skin lesion (15) sheep, (15) goats and (5) biopsy from human fingers and hand. Also, one hundred and sixteen (116) serum samples were collected from 48 sheep, 29 goats and 39 human from different ages and human from occupations workers: veterinary, veterinary technicians, shepherds, herds leader and owner ship. Eight (8) serum samples from Kids and humans with no history of vaccination against pox viruses were collected and tested for present Orf virus antibodies by ELISA as controls negative sera. The tissues suspension and serum samples stored at -20°C until used for serological and virus isolation as in Tables 1 and 2.

Negilla sativa essential oil

The Negilla Sativa essential oil cold extracted method according to the methods of Kacem and Meraihi [28], was purchased from production and marketing of medicinal plants Department and authorized by the group of Genetics and Breeding of Medicinal and Aromatic plants. Department of Genetics and cytology, Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt.

Samples preparation

The skin scarping and biopsy samples were crushed and prepared 10% suspension in phosphate buffer saline (PBS). The suspension was rapidly frozen and thawed for three successive time, then centrifuged at 3000 rpm for 15 minutes, the supernatant fluid was collected and inoculated on CAM of 11 day old ECE for three blind passages. Harvested positive CAM with pock lesion. The positive samples were inoculated into Vero cells culture for 2-3 blind passage examined daily until presence of cytopathic effect (CPE), specific CPE mainly ballooning, rounding and degeneration of cells .The isolated virus was subject to characterization and identification .

Virus purification

The isolated virus suspension from harvested positive CAM was purified by using ultracentrifugation according to the methods of [14]. Briefly, Grinding Harvested positive CAM with pock lesion was collected and centrifuged for 15 min at 3000 rpm, the supernatant was separated then re-centrifuged at 30,000 rpm/6 hrs, at 4°C the sediment was resuspended in a small volume of Tris Hcl PH 7.2 EDTA (TE) or double distal water (DDW) for E/M examination

Virus purification

The isolated viruses suspension from harvested positive CAM was purified by using ultracentrifugation according to the methods of Robinson and Petersen [14]. Briefly, Grinding Harvested positive CAM with pock lesion was collected and centrifuged for 15 min at 3000 rpm, the supernatant was separated then re-centrifuged at 30,000 rpm/6 hrs,

Species	Samples from scabs	Isolation virus on				Identification of isolated virus		
		CAM		Vero		IFAT	ELISA	PCR
		No	%	No	%			
sheep	15	3	20	2	13.33	+	+	+
goat	15	4	26.6	1	6.66	+	+	+
human	9	1	11.11	**0**	0	+	+	+
Positive control ORF virus	1	+	+	+	+	+	+	+

Table 1: Characterization and Identification of the isolated virus comparing with positive control ORF virus by FAT, ELISA and PCR.

+ = positive result

Data presented in Table 1 showed clearly that isolation of Orf virus on ECE (SPF and commercial) from prepared skin lesions (nodules, pustules, scabs and hand biopsy) from sheep , goats and human at different

localities. The results showed that the number of the clinical samples that develop positive pathological changes on CAM of ECE after the third passage were 3 samples out 15 (20%),4 samples out 15 (26.66%) and 1 samples out 9 (11.11%)respectively for sheep, goats and human clinical specimens. All samples from each of both positive and negative results on ECE were inoculated on confluent sheet of Vero cell culture. All samples which gave positive results after the third passage were two samples out fifteen (13.33%), one samples out fifteen (6.66%) and Zero samples out nine (0%) respectively gave positive results with inoculation on MDBK cell culture after the third passage. The developed CPE on Vero cells 5-7 days post inoculation appeared in the form of cell rounding, multinucleated cells, then progressing of the CPE till distortion of the monolayer and cell detachment. The Vero cell line was less sensitive than ECE for isolation and propagation of Orf virus. Identification and confirmation of isolated Orf virus was done on the molecular and biological levels. Harvested inoculated ECE positive results showed clear by FAT, ELISA, AGPT and PCR.

Location	Species	Sera	Protein A ELISA		IFAT		AGPT	
			Mean +ve ± SD	%	Mean +ve ± SD	%	Mean +ve ± SD	%
El wasta city	**sheep**	15	6 ± 0.54^{A}	40	4 ± 0.58^{A}	26.67	3 ± 0.57^{A}	20
	goat	12	4 ± 0.57^{A}	33.33	3 ± 0.57^{A}	25	2 ± 0.33^{A}	16.67
	human	11	1 ± 0.00	9.09	1 ± 0.00	9.09	0 ± 00	0
Nasar city	**sheep**	20	8 ± 0.54^{A}	40	6 ± 0.58^{A}	30	3 ± 0.00	15
	goat	8	3 ± 0.33	37.5	2 ± 0.01	25	1 ± 0.00	12.5
	human	15	2 ± 0.00	13.33	1 ± 0.00	6.66	1 ± 0.00	6.66
Baba city	**sheep**	13	4 ± 0.58^{A}	30.77	2 ± 0.00	15.38	5 ± 0.58^{A}	38.46
	goat	9	2 ± 0.00	22.22	1 ± 0.00	11.11	1 ± 0.00	11.11
	human	13	1 ± 0.00	7.6	1 ± 0.00	7.6	0 ± 0.00	0
Total ± SD		116	31 ± 0.57^{A}	26.75	21 ± 0.58^{A}	18.10	16 ± 0.58^{A}	13.7

Table 2: Examination of serum samples collected from sheep, goat and human by different serological test in different localities at Beni -Suef Governorate Egypt

A: Significant at P<0.05 SD: Stander Division

Table 2: showed clearly that the total of 116 serum samples were tested for the prevalence of anti Orf virus antibody by using different serological test protein A-ELISA, IFAT and AGPT. The percentage of positive serum samples for anti Orf virus antibody by protein-A-ELISA was (37.5, 31.03 and 10.26), IFAT were (31.03, 20.59 and 7.69) and AGPT were (10.26, 17.24 and 2.56) in sheep, goat and human in Beni-Suef Governorate respectively. We used triplicates of serum samples tested.

at 4°C the sediment was re-suspended in a small volume of Tris HCl PH 7.2 EDTA (TE) or double distal water (DDW) used for Protein A ELISA and E/M examination.

Transmission Electron Microscopic (TEM)

This method was performed according to the methods of Vikoren et al. [6]. Re-suspended of purified virus in TE buffer or DDW and stained by negative staining with 2% phosphor-tungstic acid (PH6.5) and examination by TME at Physics Department, National Research Center, Cairo; Egypt.

Fluorescent Antibody Technique (FAT)

This method was carried out according to the method of Majewska et al. [29]. Virus suspension was covered with reference rabbit anti-Orf virus antibody positive serum (Kindly obtained from Vaccine and Serum institute, Abassia, Cairo) and incubated for 30 minutes in humid chamber, washed and covered with anti-rabbit conjugated with fluoresces-isothiocyanate prepared in rabbit (Obtained from CLEVB, Abassia, Cairo).

Protein A Enzyme-linked immunosorbent assay (Protein A ELISA)

It was performed according to according to the methods of McKeever et al. [30].

Antigen preparation

The purified Orf virus was suspended in an equal volume of TNE–NP-40 (0.01 M Tris-HCl [pH 8.0], 0.1 M NaCl, 0.001 M EDTA, 1% Nonidet P-40) and used as the viral antigen. The viral antigen was diluted with 0. 10 M carbonate–bicarbonate buffer (pH 9.6), a checker board titration for assay was determined according to the methods of Azwai et al. [31].

ELISA procedure

This procedure carried out according to the procedures of Lard et al. [32]. Briefly, ELISA plate was coated with 50 µl of the antigen was dispensed into the wells of ELISA microplates. After incubation at 4°C overnight, the microplate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) (washing buffer) 3-4 time. 100 µl of diluted 1/100 sheep, goat and human serum samples were added to each well. Dilutions of negative and positive control sera were 1/100. After incubation for 60 min at 37°C after incubation wash the plate with washing buffer. 100 µl peroxidase-conjugated protein A or anti-human conjugated with horseradish peroxidase (Sigma, Aldrich, Germany) diluted with phosphate-buffered saline (PBS) containing 0.05% Tween 20. The plate was incubated further for 60 min at 37°C and then washed by washing buffer. A freshly prepared substrate solution100 µl of orthophenylenediamine (OPD), (Sigma Aldrich Germany) was added and the plates were left in the dark (10-15 min) at room temperature until color well developed in the positive control wells. The reaction was then stopped by adding 50 µl of 5% sodium dodecyl sulfate and the optical density (OD) of each well was determined with a micro-ELISA plate reader at a test wavelength of 414 nm.

Agar gel precipitation test (AGPT)

This method was performed with minor modifications of the methods of Kuroda et al. [33]. 1.5 gram agarose (Difco) and 1.5 gram glycine were added to 100 ml distal water containing 0.85 gram sodium chloride. The mixture was boiled in water bath for dissolving the agarose and left at room temperature until reach 45°C, then poured in Petri dishes 5 cm in diameter to obtained 2 mm thickness of agar. The plates were left at room temperature to solidify. After solidification of agarose in Petri dished 7 well of 3 mm in diameter were made by using metal cutter. The central well was filled with (Positive control Orf virus) and 4 peripheral wells were filled with tested sera samples. The upper and lower peripheral wells received positive and negative sera as a control. The agar palates were incubated at room temperature in a humid chamber, and examined after 24, 48 and 72 hours detecting precipitating lines.

Polymerase Chain Reaction (PCR)

DNA extraction

Samples from human and animals (Scabs lesions and biopsy samples from hand and figure) were collected for genomic DNA extraction by using DNA Mini kit (QIAGEN). 250 mg of tissue homogenized was placed in a 2 ml micro centrifuge tube, lysis buffer and proteinase K were added, and incubated at 56°C in a water bath until complete lysis of the tissues. DNA was then extracted according to the manufacturer's instructions, eluted with 100 ul elution buffer and stored at -20°C.

PCR

The B2L gene was amplified according to the methods of Inoshima et al. [24], using partial B2L gene specific primers approximately 592 bp (F:5-GTCGTCCACGATGAGCAGCT-3, R 3-TACGTGGGAAGCGCCTCGCT-5) according to Karakas et al. [27] and Zhang et al. [44]. The following reaction mixture was added in a 0.2 ml PCR tubes: DNA tamplet (100 ng/ul), 10 uL; Taq polymerase (5 U/ul), 1 ul ; 10 ul; enzyme buffer, 2 ul ; dNTP , 0.8 ul; each primer, 1 ul; added bidistilled water up to 20 ul. The mixture was briefly spine and placed in the thermal cycler, Amplification was carried out using initial denaturation at 95°C for 9 min, followed by five cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, 25 cycles of 94°C for 1 min, 55°C for 1 min,72°C for 1 min. and final elongation was performed at 72°C for 7 min. Amplified product were analyzed by agarose gel electrophoresis on 1.5% ultra pure agarose, electrophoresis grade (Gibco BRL), containing 0.5 ng /ml ethidium bromide in TAE buffer and visualized on an UV transilluminator. A 900–100 bp DNA ladder (Finzyme, Finland) was used as marker.

Antiviral activity of essential oil determined by Pock Reduction Assay

Virus titers were estimated from cytopathogenicity by tenfold dilution method 0.02 ml of each dilution was inoculated in five CAM of ECE for each dilution and expressed as 50% egg infectious dose per ml (EID50/ml and calculation was performed according to Reed and Munch [34].

Statistical Analysis

All data were subjected to statistical analysis including the calculation of Mean, Stander Division. Significance between data was evaluated by the Student *t*-test at level *P or t*<0.05 according to Petrie and Watson [35] using version 15 of SPSS computing program (SPSS for Windows, version 15; SPSS, Chicago).

Results

Virus isolation on CAM of ECE

Scabs and biopsy samples were inoculated on chorioallantoic membranes (CAM) of ECE, characteristic pathological changes

edema, thickening, hemorrhages, and small grayish white foci considered positive result. Recovery virus was harvested from CAM, after adaptation inoculated isolated virus on CAM for 3 passage until clear cytopathic effect (CPE). The positive percent of inoculated skin scab and biopsy with specific changes pock lesion in CAM were (3/15, 20%), (4/15, 26.6%) and (1/9, 11.11%) from sheep, goats and human respectively. as in Table 1 and Figure 3.

Detection of Orf virus antibody

The Data presenting in Tables 2 and 3 and Figures 1 and 2

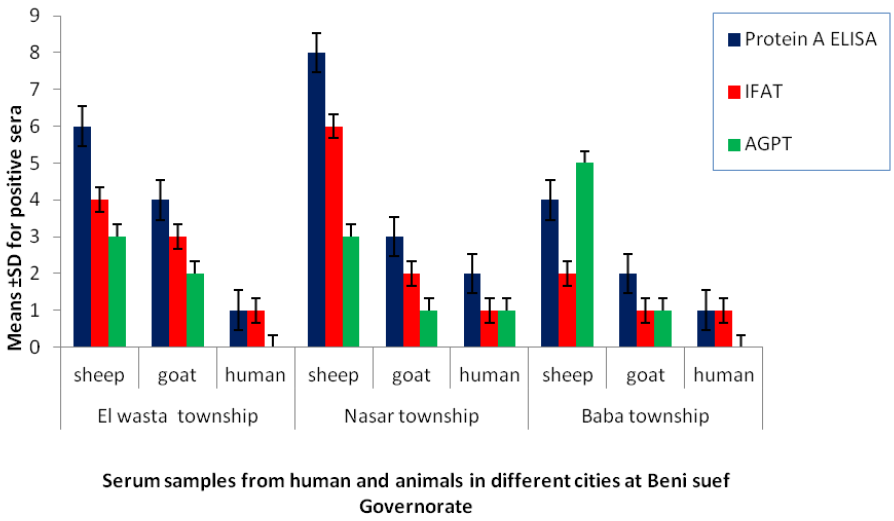

Figure 1: Determination of Orf virus antibody in serum samples of sheep, goat and human by ELISA, IFAT and AGPT in different localities.

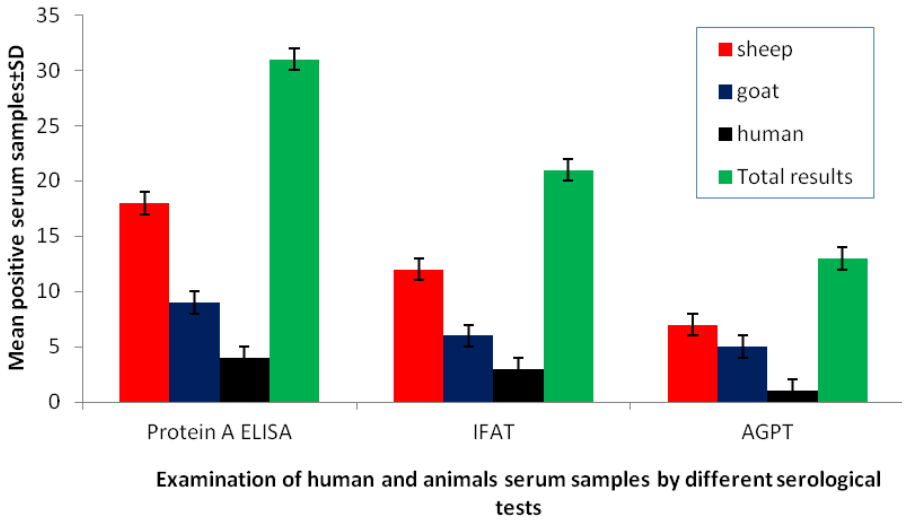

Figure 2: Total positive percent of anti Orf virus antibody in human and animals serum samples by different serological tests. When Comparing between protein A ELISA, IFAT and AGPT in detection of ORF virus infection in human and animals according to mean positive serum sample showed clearly that the ELISA more accurate and sensitive than IFAT and AGPT in detection of ORF virus antibodies.

Species	Total of serum samples	Protein A ELISA		IFAT		AGPT	
		Mean +ve ± SD	%	Mean +ve ± SD	%	Mean+ve ± SD	%
sheep	48	18 ± 0.59 ^{A}	37.5 ^{A}	12 ± 0.57 ^{b}	25 ^{b}	7 ± 0.56 ^{C}	14.5 ^{C}
goat	29	9 ± 0.55 ^{A}	31.03 ^{A}	6 ± 0.54 ^{b}	20.69 ^{b}	5 ± 0.59 ^{C}	17.24 ^{C}
human	39	4 ± 0.57 ^{A}	10.26 ^{A}	3 ± 0.54 ^{b}	7.69 ^{b}	1 ± 0.00 ^{C}	2.56 ^{C}
Total results	116	31 ± 0.59 ^{A}	26.72 ^{A}	21 ± 0.88 ^{b}	18.10 ^{b}	13 ± 054 ^{C}	11.21 ^{C}

A,B and C: High significant at P<0.05 by using t test CI=95 %
A: Significant at P<0.05 , B: Significant at P < 0.05, C: Significant at P <0.05
SD: Stander Division

Table 3: Examination of serum samples collected from animals and human by different serological test.

showed clearly that the total of 116 serum samples were tested for the prevalence of anti Orf virus antibody by using different serological test protein A-ELISA, IFAT and AGPT. The tested positive serum sample as well reference anti Orf virus gave ODs more than threefold of negative control serum samples. The percentage of positive serum samples for anti Orf virus antibody by protein-A-ELISA were (37.5, 31.03 and 10.26), IFAT were (31.03, 20.59 and 7.69) and AGPT were (10.26, 17.24 and 2.56) in sheep, goat and human in Beni-Suef Governorate respectively.

Data presented in Figure (1 and 2), showed that the total percentage of positive serum samples were (26.72, 18.19 and 11.21) determined by protein-A-ELISA, IFAT and AGPT, there was a significant difference ($p<0.05$) between all tests used. Also, the results showed clearly that the seroprevalence of anti-ORF virus antibodies among sheep, goat and human tested by ELISA in El Wasta were (40%, 33.33% and 9.09%) , Nasar were (40%, 37.5% and 13.33%) and Beba were (30.77% , 22.22% and 7.6%), by IFAT in El Wasta were (26.67%, 25% and 9.09%) , Nasar were (30%, 25% and 6.6%) and Beba were (15% , 11.11% and 7.6%) and by AGPT in El Wasta were (20%, 16.7% and 0%), Nasar were (15%, 12.5% and 6.6%) and Beba were (38.46% , 11.11% and 0%) sheep , goat and human respectively.

Virus characterization

Examination of purified virus suspension stained with negative stain by transmission electron microscope (TEM) showed ovoid shape particles 290-300 × 160 nm in diameter characteristic ball of wool appearance closely similar to the particles of reference Orf viruses virus as in Figure 5. The isolated virus suspension was identified by direct FAT with control positive anti Orf virus antiserum obtained from (Vaccine and Serum institute, Abbassia, Cairo) which showed specific greenish fluorescence, similar to reference Orf virus suspension while the non infected one was free from any fluorescence. The isolated virus suspension was confirmed with direct ELISA, the positive results give ELISA ODs more than three ODs of control negative samples, the isolated virus give nearly similar to reference Orf virus and confirmation of isolated virus by PCR with partial B2L gene, PCR product approximately 592bp product for B2L gene (envelope gene) fragments of Orf virus DNA were typical to reference Orf virus strain in human and animals. Electrophoresis analysis Lane M: Marker 100 bp Lane 1: control positive reference Orf virus 592 bp. Lane 2, 3 and 6: positive samples from human and animals samples. Lane 4, 5 negative samples Lane 7: PCR control negative using water as a template Figure 5.

Antiviral activity

The essential oil was observed safe, no effect in the nature and color of embryo fluid comparing with control. It was reduced infectivity titer of treated Orf virus with essential oil from $\log_{10}$ 5.9 to 1.2 within 40

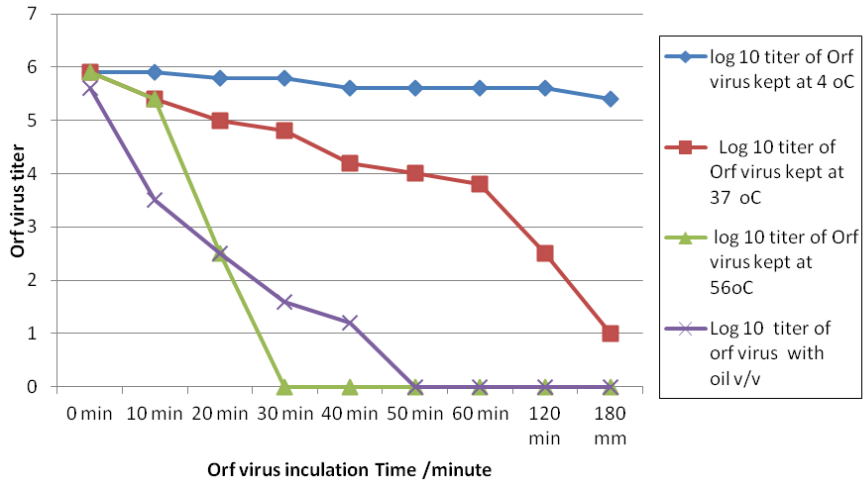

Figure 3: Reduction of ORF virus titer treated with essential oil kept at 37°C. Orf virus isolate was held at 37°C, a 1.1 Log units decrease in the titer was evidenced by the 30 minutes but virus held at 56°C, was much accelerated decrease 3.4 Log units being inactivated within the first twenty minutes until complete inactivation within 30 minutes. Essential oil mixed with virus suspension (v/v) at 37° C decrease viral activity 3.1 Log units this reduction due to effect of 37C and essential oil.

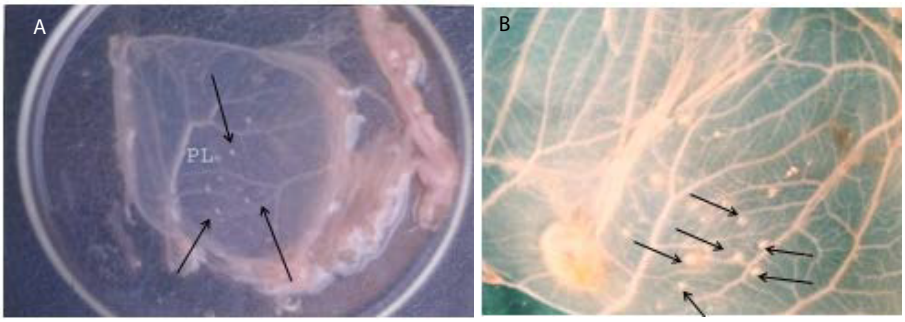

Figure 4: Chorio-allantoic membranes of the ECE inoculated with ORF virus produced the characteristic pock lesions (black arrows). Fig 4(A) very small pock lesion, Fig 4 (B) well developed pock lesion.

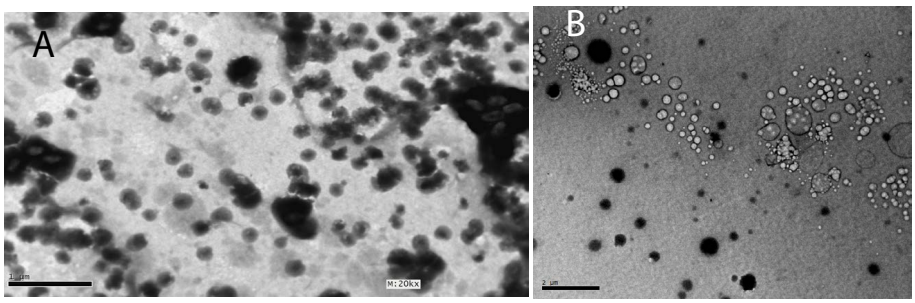

Figure 5: A: Eelectron micrograph of purified suspension from inoculated CAM infected materials, showed oval-shaped virus particles (blue arrows). The virus particles are approximately 290-300×160 nm in size. **B:** Negative CAM suspension showed no virus particles.

minutes comparing with virus titer kept at 37°C without oil as in Table 3 and Figure 3.

Discussion

Contagious ecthyma is a serious threat to the healthy development of the sheep, goat and human public health hazard, early identification of Orf virus is effective method for prevention and control of Orf virus infection [3]. Mouth sores is a painful skin disease caused by ORF virus characterized by developing of scabby lesions around the mouth and nostrils of sheep and goat worldwide distribution [36]. Orf lesions spontaneously regressed in all affected sheep and goat within 4 weeks but in some cases caused severe proliferative dermatitis and sometimes deaths [10]. In the present study increasing number of Orf virus infection in sheep and goats in Egypt may be due to absence of vaccination strategy and continuous moving infected sheep and goat flocks between different grazing area which spread Orf virus-rich scabs shed by the previous year's infected animals or carrier animals play an important role in the spread of Orf virus outbreaks, this observation is inagreement with [10,37]. It has long been accepted that ORF virus infections in sheep and goat provide no long-lasting immunity, and annual outbreaks in flocks are relatively common as reported by [38].

Skin scabs, biopsies and serum samples were collected from sheep, goats and human in Beni Suef Governorate during 2013 outbreak for virus isolation, identification and serological diagnosis of Orf virus. Although the disease could be readily diagnosed by clinical signs but it need more confirmation by different laboratory tools as FAT, ELISA, E/M and PCR. Also, differentiated Orf virus than other diseases which produce similar clinical findings such as; sheep pox, foot and mouth disease (FMD), bovine herpes virus type-2 and bluetongue virus [5-7,22]. The prepared suspected samples were inculcated on CAM of ECE developed the characteristic pock lesions as pathological change characteristic of Orf viruses as Figure 4 and this finding is inagreement with [4,10]. ECE is easy, simply and rapidly of use for virus adaptation within short time. Wherever, using Vero cells culture need 21 days passages for virus adaptation CPE developed as in Tables 1 and 2, Figures 4 and 5 this finding is in agreement with [6].

Electron microscope (E/M) has an increasingly useful tool in the viral diagnostic field not only for a primary diagnosis but also confirmation ORF virus. Examination of purified isolated viruses by E/M ,micrograph showed ovoid shape particles 290-300×160 nm in diameter as bull of wool similar to morphology of reference ORF virus particles as in Figure 5 this result is similar finding by [22,39]. Although virus isolation is considered a gold standard for Orf virus detection but electron microscopy is a benchmark method for confirmation of the virus, but time consuming, laborious and lack of sensitivity in some samples which lead us to search other easy and simple confirmatory tests as reported by [32].The identification and characterization of isolated ORF virus on the basis of clinical signs, E/M , IFAT, ELISA and PCR which give similar result to the Egyptian Orf virus this result is inagreement with [11]. Interestingly, results that the isolated ORF virus from human is identical of Orf virus isolated from sheep and goat in Egypt as in Figures 4-6. PCR is a rapid, sensitive and specific tool in identifying several infectious diseases of veterinary importance as mention by [40-43]. Comparing different serological tests AGPT and IFAT with ELISA for detection of anti Orf virus antibodies. The total positive percent of serum samples were (11.21%, 18.10% and 26.72%) and t there was a significant difference ($p<0.05$). ELISA was found simples, sensitive and accurate for rapid diagnosis of Orf virus in human and animals in Egypt. Increasing the positive percent of Orf virus antibodies in the serum samples of human and animals may be due to cross reactivity with viruses belong family family Poxviridae this finding is in agreement with that obtained by [4,10].On the other hand this finding is disagreement with [42-44].They found that cross immunity between orthopoxvirus and ORF virus do not occur and the

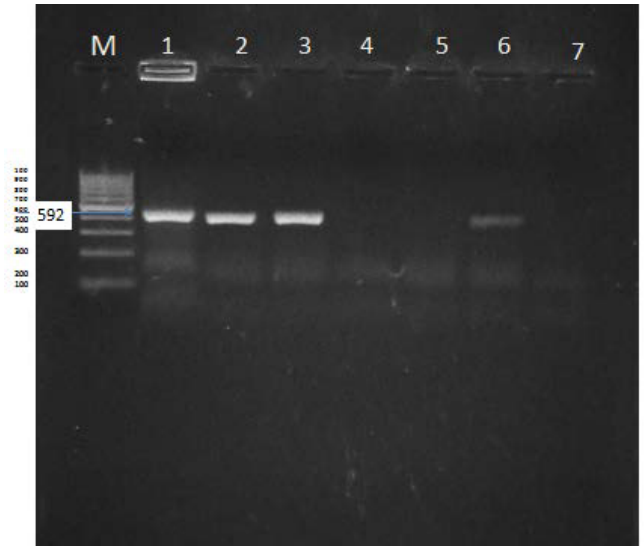

Figure 6:. Electrophoresis analysis 1.5 agarose of DNA amplified fragment from ORF virus B2L gene primer at 592 bp . DNA was amplified by PCR for the detection ORF virus in clinical samples and confirmation of isolated virus. Lane M: Marker 100 bp Lane 1: control positive reference ORF virus 592 bp. Lane 2,3 and 6 : positive samples from human and animals samples . Lane 4,5 negative samples Lane 7: PCR control negative using water as a template.

Time	Log $_{10}$ ORFV titer kept at 4 °C	Log $_{10}$ ORFV titer kept at 37 °C and 56°C		Log $_{10}$ ORFV titer was treated with essential oil (v/v)	
		Log$_{10}$ 37 °C	Log$_{10}$ 56°C	Log $_{10}$ titer	Reduction index
0 min	5.9	5.9	5.9	5.6	0.3
10 min	5.9	5.4	5.4	3.5	2.4
20 min	5.8	5.0	2.5	2.5	3.4
30 min	5.8	4.8	0	1.6	4.2
40 min	5.6	4.2	0	1.2	4.4
50 min	5.6	4.0	0	0	5.6
60 min	5.6	3.8	0	0	5.6
120 min	5.6	2.5	0	0	5.6
180 mm	5.4	1.5	0	0	5.4

Table 4: Antiviral effect of essential oil on ORF virus titer by pock reduction test.

(Table 4) showed clearly that the Orf virus isolate was held at 37°C, a 1.1 Log units decrease in the titer was evidenced by the 30 minutes, then, gradual decreasing until complete inactivation. When the viral isolate was held at 56°C, the decrease in viral activity was much accelerated, with a bulk of the virus 3.4 Log units being inactivated within the first twenty minutes and the complete inactivation was achieved within 30 minutes. Essential oil mixed with virus suspension (v/v) decrease viral activity 3.1 Log units complete inactivation was achieved within 40 minutes.

diseases caused by Orf virus species are identical in animals & humans. Antiviral effect of essential oil may be due to block the viral infection by blocking the cell membrane receptor for Orf virus or induce internal changes in the host cells, which in turn affect the virus replication cycle or due to production cytokines which blocked viral infection other health cells as in Table 4 and Figure 3. This result was in agreement with [44]. They found the essential oil caused sharp reduction in small pox virus infectivity titer. Also, they observed that the concentration of virus was reduce $\log_{10}$ 5-1 and they infectivity titer were reduced 21 and 25 time more than control group. We found that the essential oil reduced plaque size and number when mixing with Orf virus before inoculation on CAM. Finally, Our results indicated that PCR and ELISA are rapid, sensitive and specific tool to identify ORF virus infection in human and animals and Negilla sativa essential oil may be use as antiviral.

Conclusion

In conclusion, this study indicates that Orf virus infection occurs in sheep and goat and infected human who direct or indirect contact with infected animals in some Egyptian Governorates. Orf virus is preferably isolated in CAM of ECE than Vero cell. Moving infected sheep and goat with ORF virus to place other than reared place or grazing area causes introduced Orf virus infection into new area .It is recommended that conduct field serological tests and vaccination strategy should be carried out in sheep and goat on a regular basis to avoid potential risk factors and cut a link in the transmission chain of zoonotic infections. The essential oil inoculated ECE without virus was observed safe, no effect in the nature and color of embryo fluid comparing with control negative. Negilla sativa essential oil mixed with Orf virus, it reduced infectivity titer of treated Orf virus with essential oil from $\log_{10}$ 5.9 to 1.2 within 40 minutes comparing with virus titer kept at 4°C and 37°C without oil.

Disclosure Statement

Conflict of Interests

The authors do not have any conflict of interests regarding the content of the paper.

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