Global Veterinaria 12 (1): 19-25, 2014
ISSN 1992-6197
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DOI: 10.5829/idosi.gv.2014.12.01.81156

Isolation of Parapoxviruses from Skin Lesion of Man and Animals in Middle Egypt

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Abstract: Parapoxviruses (PPVs) are important in various animals as well as in humans as zoonotic infections. Reliable detection of Parapoxviruses is fundamental for the exclusion of other rash-causing illnesses. Biopsy (vesicles and scabs) samples from infected 11 cattle and 8 human (5 dairy farmers & 3 veterinarians) in Middle Egypt (Beni-Suef and El-Fayoum Governorates) were inoculated on chorio-allantoic membrane (CAM) of specific pathogen free (SPF) and/or commercial embryonated chicken egg (ECE) for three blind passages. The isolated virus induced pock lesions on CAM. Electron microscopy examination of purified isolated virus suspension by negative staining technique demonstrated 290-300×260 nm virion very similar to PPVs. Confirmation of isolated parapoxvirus was achieved by appearance of a clear precipitating line by AGPT and by ELISA comparing reference PPV strain which indicated that the given samples contain parapox viral antigen. A total of 90 serum samples from 75 cattle (45 cows & 30 buffaloes) and 15 human was tested for antibodies against parapoxvirus using ELISA. Overall seroprevalence among cows, buffaloes and human were 12/45 (26.6%), 4/30 (13.33 %) and 3/15 (20 %), respectively. The prevalence rate was higher (32, 20 and 22.2%) in Beni-suef Governorate than (20, 10 and 16.6%) in El-Fayoum Governorate among cows, buffaloes and human, respectively. Our results in this study suggested that Parapoxviruses infection spread among the population of Egyptian cattle and human in Middle Egypt. Lack of vaccination lead to appearance of clinical disease in different parts of Egypt, it is supposed that Parapoxviruses is becoming enzootic in Egypt and so proper hygiene and health education may be useful in the prevention of zoonotic infections with any of these viruses.

Key words: Parapoxviruses · CAM · Virus isolation · Electron Microscopy (EM) · ELISA · Middle Egypt

INTRODUCTION

Poxviridae are a family of oval or brick-shaped, quite large, double-stranded DNA viruses that can infect both humans and animals. The genus Parapoxvirus (PPVs) is included among these viruses; it measures 260 X 160-nm and possesses a unique spiral coat that distinguishes them from the other poxviruses [1]. Parapoxvirus species which are enzootic to hoofed animals (Ungulates) throughout the world consists of five similar species; Bovine papular stomatitis virus (BPSV), orf virus (ORFV), parapoxviruses of red deer in New Zealand (PVNZ), pseudo cowpox virus (PCPV) and squirrel parapoxviruses (SPPV); as well as three tentative species; auzdyk disease virus, ecthyma contagiosum virus (ECV) and seal pox virus [2].

Parapoxviruses cause papules, nodules and scabs in the skin around the lips, nose, mouth and teats of affected animals such as sheep, goats and cattle [3]. In some severe cases, the papules/vesicles progress to pustules and ulcers. PPVs are highly contagious, occasionally infect humans by close contact with the skin lesions of infected animals or indirectly transmitted through the handling of virus contaminated materials. The zoonotic hosts of these Parapoxviruses are sheep and goats (ORFV and ECV) and cattle (PCPV and BPSV) which cause

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a disease known as milker's nodule in human and the infections are therefore known as zoonoses [4, 5]. Among high-risk populations, such as animal caretakers or meat handlers, the typical clinical appearance and the benign nature of the infection may be well known. As a result, infected individuals may not seek medical attention and many authors believe that the infection is much more common than actually reported [6].

Histopathologically, Parapoxvirus infections are indistinguishable from one another; epidermal hyperplasia, mild acanthosis, spongiform keratinocytic degeneration and viral cytopathic changes occur, including cytoplasmic inclusion bodies and nuclear and cytoplasmic vacuolization [7, 8]. Laboratory diagnosis of Parapoxviruses achieved by negative-stain electron microscopy (EM) from scabs of affected skin tissue allows direct visualization of the Parapoxviruses; identification is based on the characteristic ovoid cross-hatched appearance of the virion [9]. The development of PCR methods for detection of Parapoxviruses has met the demands for specific and sensitive laboratory diagnosis of Parapoxviruses [5]. However, the lack of an electron microscope and PCR in many diagnostic laboratories need to develop reliable serological and immunological diagnostic tests for Parapoxviruses detection [10, 11]. The diagnosis of Parapoxviruses may be confirmed even after skin lesions have healed by serologic testing; Enzyme linked immunosorbent assays (ELISA) and agar gel precipitation test (AGPT) of patient's serum but these tests are unable to distinguish the specific species [12].

Recently a disease characterized by papules, nodules, vesicles and pustules, which sometimes progressed to ulcers on teats has been seen among individual sheep, cows, buffaloes, camels and also human skin lesion in different governorates of Egypt [13, 14]. So in this study we had collected lesion materials for isolation of the virus from those affected cows and buffaloes and also, biopsy from human lesion. Virological and serological investigations were carried out to identify the isolated virus.

MATERIALS AND METHODS

Clinical Cases:

Animals: During summer 2012, 11 cases of 6 cows and 5 buffaloes from different localities in Beni Suef and El Fayoum Governorates, suffering from papules, nodules, vesicles, pustules and ulcers on teats and udder as well as drastic drop in milk production were submitted to the veterinary clinic for clinical examination. The affected animals had mild increase in the body temperature. The course of the infection took around 15 days and after 28 days the animals recovered from clinical disease.

Humans: Characteristic clinical signs of milker's nodules were observed in 8 persons; 5 farmer and 3 veterinarian contacts with infected cattle that had severe papules/vesicles progressed to pustules and ulcers in their udder. They had proliferative lesions and scabs on their hands. All the lesions observed in humans healed completely within 4 to 5 weeks of their onset.

Tissue and Blood Samples: Tissues (Biopsies) from lesions of infected 6 cows and 5 buffaloes and 8 human (5 dairy farmers & 3 veterinarians) were collected. In addition, a total of 90 blood samples from 45 cows and 30 buffaloes and 15 human (12 dairy farmers & 3 veterinarians) in Middle Egypt (Beni-Suef and EL-Fayoum Governorates) was collected. Tissue samples were triturated in phosphate buffered saline (PBS-pH 7.4) and a 10% (w/v) suspension was made for virus isolation and sera were prepared and stored at -20°C until serological tests were done

Virus Isolation in Embryonated Chicken Eggs: Commercial and SPF embryonated chicken eggs (ECE) obtained from a commercial company and hatching laboratory in Beni-Suef and El-Fayoum Governorates, Egypt; respectively were used for virus isolation. Crusted scab lesions and skin biopsies were ground making up a 10% (w/v) solution; antibiotics mixture (penicillin 100 U/ml and streptomycin 100 ng/ml) was added. The suspension was frozen at - 20°C and thawed three times, after centrifugation at 3000 rpm / 15 min. 0.2ml of the supernatant was inoculated onto CAM of ECE, eggs were incubated at 37°C for a period of 3-5 days after that CAM was examined for detection of virus lesions [15]

Agar Gel Precipitation Test (AGPT):

Control Virus and Positive Serum: Reference Parapoxviruses (orf vaccine,Scabivax UK) strain were and control positive anti- PPV serum (CLEVB, Abbassia, Cairo)used as controls.

AGPT Procedure: The AGPT tests were performed with minor modifications of the method reported by Kono *et al*. [16]. 075 gram agarose (Difco) and 0.75 gram glycine were added to 50 ml distal water containing 0.425 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 obtain 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 PPV hyperimmune serum) and 4 peripheral wells were filled with tested antigen samples. The upper and lower peripheral wells received positive and negative antigen as a control. The test was usually carried out in reverse manner for detaching the antibodies. The central well was filled with a Reference PPV antigen and the 4 peripheral wells received positive and negative control sera. The agar palates were incubated at room temperature in a humid chamber and examined after 24, 48 and 72 hours for detecting precipitating lines.

Enzyme-linked Immunosorbent Assay (ELISA): Antigen preparation: The grown Pock lesions of Parapoxviruses on CAM were harvested by 3 successive cycles of freezing and thawing 5 days after inoculation and then used to prepare ELISA antigen according to the procedure mentioned by Azwai *et al*. [10], the virus suspension was centrifuged at 3000 rpm/ 15 min. Supernatants containing the virus were collected and pelleted by ultracentrifugation at 25,000 rpm / 60 min at 4°C and then pellet was re-suspended in PBS and its protein content was determined by the method of Lowry *et al*. [17] and stored at -70°C until being used for ELISA.

ELISA Procedure: Standard methods for ELISA were carried according to Gokce and Woldehiwet [18] with some modifications to detect anti-orf virus antibodies or confirmed PPV antigen. Briefly, ELISA plates were coated with 100 µl of previously prepared viral antigen. The ELISA plates were incubated in a moist chamber at 37°C for 1 h, then overnight at 4°C. Unbound antigen was washed off with washing buffer, three times After washing blocking plate with 100 ul of 1% bovine serum albumin [BSA, Sigma/PBS/Tween 20 (0.05%)], plates were incubated for 30 min in a moist chamber at 37 oC then washed. 100 µl of diluted 1/50 cattle or human serum samples were added to each well in duplicate. Dilutions of known negative and positive control sera were also added in duplicate. After incubation for 60 min at 37°C and washing, 50 µl of dilution 1/1000 of anti-bovine (Sigma) or anti-human conjugated with horseradish peroxidase (Sigma, Aldrich, Germany) was added. The plates were incubated further for 60 min at 37°C and then washed. A freshly prepared substrate solution100 µl of ortho-phenylenediamine, (OPD, Sigma Aldrich Germany) was added and the plates were left in the dark (10-15 min) at room temperature until color had developed in the positive control wells. The reaction was then stopped by adding 50 µl of 2.5 M H2SO4 and the optical density (OD) of each well was determined with a micro-ELISA plate reader at a test wavelength of 450 nm. The mean OD of the control negative serum plus 3 times the standard deviation was regarded as the cut-off value for the assays. OD values of the test serum samples equal or greater than the cut-off value were considered positive [19].

Electron Microscopic Examination (EM): Grinding chorio-allantoic membranes that showed pock lesions were collected and centrifuged for 30 min at 3000 rpm, the supernatant was separated then re-centrifuged at 20000 rpm / 2 hrs, the sediment was re-suspended in distilled water and prepared by negative staining with 2% phosphotungstic acid (pH 6.5) [20]. Examination by E/M was carried out at Physics Department, National Research Center, Cairo; Egypt.

RESULTS

Virus Isolation: Tissue specimens (Homogenized vesicles) from cattle and human with clinical symptoms showed positive pathological changes on chorio-allantoic membrean (CAM) of embryonated chicken eggs (ECE) after three blind passages. Egg inoculation detected PPV in 6/19 (3 from 6 cows, 1 from 5 buffaloes and 2 from 8 human) (31.5%) samples from the infected animals and human with production of pock lesion on the chorio-allantoic membranes (Table 1 & Fig. 1).

Identification of the Viral Agents by AGPT and Confirmed with ELISA: The isolated virus strain beside the reference one were clearly identified by AGPT and confirmed with ELISA. The isolated viruses showed clear precipitating line in AGPT with positive PPV hyper-immune serum and ELISA OD Means ±SD were 0.518±.040, 0.486±0. 027 and 0.639 ±0.031 for isolated virus from scabs samples from cattle, skin scrap and skin biopsy from human and Reference PPV propagated on CAM respectively. All of them were more than cutoff value. This result showed clearly that the isolated viruses resembled stander PPV (Table 2)

Table 1: Results of inoculation on CAM of ECE after three passages

	Cows			Buffaloes			Human		
Location	No	+ve	%	No	+ve	%	No	+ve	%
Beni - Suef Governorate	3	2	66.6	2	0	0	5	1	20
El-Fayoum Governorate	3	1	33.3	3	1	33.3	3	1	33.3
Total	6	3	50	5	1	20	8	2	25

+ ve : Positive samples

Table 2: Identification of the isolated viruses by AGPT and confirmed by ELISA assays

Isolate	Harvested CAM	AGPT	ELISAMean ±SD
1. Scabs samples from cattle	Pock lesion ++	+PPL	0. 518±.040*
2.Skin scrap and skin biopsy from human	Pock lesion ++	+ PPL	0. 486±0. 027*
3. Reference PPV propagated on CAM	Pock lesion ++	+ PPL	0.639 ±0.031*

++ pock lesion on CAM of ECE + clear perception line on AGPT

* Results of positive samples from cattle and human gave similar results as Reference PPV ELISA OD mean ±SD = cutoff value

Table 3: The Prevalence of Parapoxvirus infection among cattle and human at Beni-Suef and El-Fayoum Governorates (Middle Egypt) tested by ELISA

	Cows			Buffaloes			Human		
Location	No	+ve	%	No	+ve	%	No	+ve	%
Beni-Suef Governorate	25	8	32	10	2	20	9	2	22.2
El-Fayoum Governorate	20	4	20	20	2	10	6	1	16.6
Total	45	12	26.6	30	4	13.3	15	3	20

+ve: Positive samples

Fig. 1: Chorio-allantoic membranes of the ECE inoculated with PPV samples on CAM produced the characteristic pock lesions of Poxviridae family (black arrows)

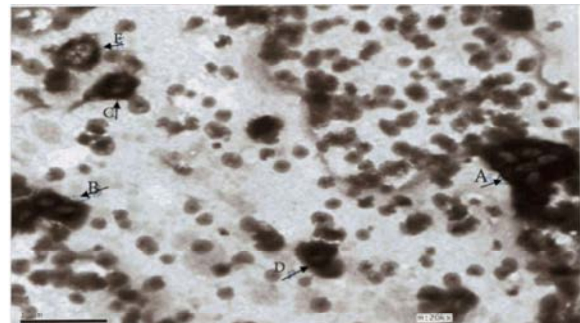

Fig. 2: Eelectron microphotograph of purified suspension from infected materials, showed oval-shaped virus particles with the arrangement of the outer surface protein filaments (black arrows with capital letters A, B, C, D & E). The virus particles are approximately 290-300 ×160 nm in size.

Electron Microscopic Findings: The typical parapoxvirus particles were identified in pock lesions of the fresh purified infected CAM stained with negative stain and examined by E/M. The virus particles were ovoid, >290 × 160 nm in size, with a surface filament (Figure 2).

Serological Findings: The overall seroprevalence of anti-parapox virus antibodies among cows, buffaloes and human from Middle Egypt (Beni-suef and El-Fayoum Governorates) tested by ELISA were 12/45 (26.6 %), 4/30 (13.33 %) and 3/15 (20 %), respectively. Higher infection rates (32, 20 and 22.2%) were detected in Beni-suef Governorates than (20, 10 and 16.6%) in El-fayoum Governorates among cows, buffaloes and human, respectively Table (3).

DISCUSSION

Contagious erythema, caused by parapox virus is one of the most common skin diseases of ruminants and they are the main hosts that become infected with PPV worldwide. Affected animals such as cows and buffaloes

develop proliferative dermatitis in the region of teats and udder skin. In young, stressed, or immunosuppressed cattle formation of severe bloody lesions were detected [15, 21].

The most effective way for orf virus to enter into a new area is by introduction of infected animals. In addition, infections of humans by PPV occur after direct contact with lesions of infected animals. In our results up to 20% in individuals at risk (Had antibodies against parapox virus) (Table 3), this finding nearly similar with 22% that reported by Robinson and Mercer [8] in human.

The well-known typical milker's nodule occurs most commonly on the index finger of a farmer and veterinarian characterized by lesion resembling a tumor and resolves spontaneously, usually without complications. This finding is in agreement with that found by Ghislain *et al*. [22].

In the present study, parapoxvirus was isolated and identified as a member of the genus parapoxvirus in the family Poxviridae on the basis of clinical signs, AGPT, E/M and confirmed by ELISA; accordingly, the Egyptian strain would be a pseudocowpox virus or milker's nodule virus (Tables 2). These identification procedures are compatible with those methods reported for virus isolation on primary lamb testis cell culture [23] and for isolated bovine parapoxvirus on fetal bovine lung [24]. Previous investigations reported the possibility of isolation of parapoxvirus on ECE revealed that primary isolation with blind passage required usually 12 days [25, 26]. However, in the current study the virus isolation was successful in CAM of SPF-ECE and also in commercial ECE with isolation procedure of 5 days incubation and clear small pock lesions appeared after the 2nd or 3rd passage (Table 1 and Figure 1).

The isolated virus strain beside the reference one were clearly identified by AGPT and confirmed with ELISA. The isolated viruses showed clear precipitating line in AGPT with positive PPV hyper-immune serum and ELISA OD Means ±SD were 0.518±.040, 0.486±0.027 and 0.639 ±0.031 for isolated virus from scabs samples of infected cattle, skin scrap and skin biopsy of infected human and Reference PPV propagated on CAM respectively. All of them were more than cutoff value. This result showed clearly that the isolated viruses resembled stander PPV lesion (Table 2 Figure 2).

Electron microscope technique has become an increasingly useful tool not only for reaching a primary viral diagnosis but also for corroborating the findings produced by more recent techniques [16]. However EM is rapid and clearly distinguishes PPV particles. It requires high viral loads (10 6 particles/ml) of fresh lesions and crusts of infected individuals to avoid false-negative results in cases of lower viral loads [27, 28]. In our study we were able to show classic ovoid 290-300×160nm in diameter and slightly crisscross spiral pattern of the virions by negative-staining electron microscopy (Figure 2) which may confirm a parapox virus infection but cannot distinguish orf virus from other parapox viruses such as pseudocowpox; this finding is nearly similar with all published cases of PPV infection diagnosed by EM [29, 30,]. Virus isolation is considered a gold standard for PPV detection and electron microscopy is a benchmark method for confirmation of the virus.

There is no report which describes a serological survey of parapoxviruses in Egypt. In the current study the sero-prevalence rate of anti parapoxviruses antibodies by ELISA was 26.6, 13.3 and 20 % among cows, buffaloes and human, at middle Egypt respectively (Table, 3).

Our finding is slightly is in agreement with that obtained by Azwai *et al*. [10], Czerny *et al*. [11], Yirrell *et al*. [31], Tantawi [32] and Lederman *et al*. [33]. They also found that cross immunity between orthopoxvirus and parapoxvirus do not occur and the diseases caused by parapoxvirus species are identical in animals and humans and so accordingly the Egyptian isolates of the parapoxviruses isolated from all the animals and human were identical.

Instruct patients with close direct contact with sheep, goats, or cattle to use proper hand hygiene and personal protective equipment (protective gloves) to decrease the risk of infection. This is especially crucial when in contact with herds during an infection outbreak of any of these Parapoxviruses or following vaccination with the live attenuated orf virus vaccine [33]. Several common disinfectants effectively reduce the numbers of virus particles present on surfaces and their use may help to prevent spread of the disease during outbreaks. In addition, infected animals should be isolated from the rest of the herd and immunocompromised individuals should avoid contact with infected animals [34].

The data obtained in this study reported successful isolation of Parapoxviruses (PPV) in ECE by CAM route in 3-5 days confirmed by AGPT, E/M and ELISA which are considered as powerful tools for the diagnosis of Parapoxviruses and can differentiate it from poxvirus infections. Using ELISA for the detection of antibodies against PPV from human and animals population is a very useful method for sero-epidemiological surveys. Also this study supposed that the Parapoxviruses is becoming

enzootic in Egypt and proper hygiene and education required for the prevention of the zoonotic infections with any of these viruses.

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