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Adaptation of Capripox Virus Isolate from Goats in Heterologous Cells

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ABSTRACT

An outbreak of goatpox was attended in district Durg and surrounding regions of Chhattisgarh state. Dried skin scabs were collected from 250 goats of different age groups showing clinical signs suggestive of pox. The prevalence rate was studied based on severity of clinical signs followed by confirmation with agar gel immuno diffusion (AGID) test. Positive scabs were further processed for virus isolation in embryonated chicken eggs via dropped chorio allantoic membrane (CAM) route followed by inoculation of CAM material in chinese hamster ovary and chicken embryo fibroblast cell cultures and propagated up to fifth passage level. Goatpox was reported with a prevalence rate of 74% using AGID. Distribution of disease in young animals, particularly in the kids (below 6 month) was more (45.4%) as compared to those between 6 to 24 months (31.35%) and 24 months and above (23.24%). Capripox virus showed cytopathic effect in chinese hamster ovary cells only after third passage and no cytopathic effect in chicken embryo fibroblast cells even up to fifth passage. Cytopathic effects appeared after 72 hrs in chinese hamster ovary cell and were characterized initially by rounding and clumping of cells, cytoplasmic vacuolation followed by cell detachment. Identity of capripox virus was confirmed positive in both the cultures from third to fifth passage by both AGID and countercurrent immuno electrophoresis (CIE) test. In conclusion, field isolate of capripox virus was successfully adapted and propagated on chinese hamster ovary cell following third passage.

Keywords: Goatpox, Capripox virus, Prevalence, Chinese hamster ovary, Chicken embryo fibroblast

Goats occupy a premier place in the livestock industry and contribute significantly to the world economy. India ranks second in the world goat population with 135.17 million sharing more than 20 % of global goat population (Livestock Census, Anon. 2012). The total goat population in Chhattisgarh is more than 3.2 million that comprises 21 % of the total livestock population in the state. The populations of goats are threatened by a number of health hazards, among the most notable of which is goatpox. Outbreaks of goatpox are being reported in different regions of India *viz.* Himachal Pradesh (Verma *et al*. 2011) and Maharashtra (Kadam *et al*. 2014) as well as in Chhattisgarh (Joshi, 2000). It is malignant pox disease of goats caused by *Capripoxvirus* of the family *Poxviridae* (Murphy *et al*. 1995). Diagnosis of goatpox is usually based on clinical signs followed by laboratory confirmation. Embryonated chicken eggs (Kadam *et al*. 2014) and primary cell cultures like lamb kidney and testes (OIE, 2012) are popular cultivation system used for sheeppox and goatpox (capripox) viruses. However, capripox virus can be successfully adapted in chicken embryo fibroblast (CEF) cells (Rao and Malik, 1982). Chinese hamster ovary (CHO) cells are one of the main cell line used in the production of

recombinant therapeutics (Wurm, 2004). Although, CHO cells have restricted virus susceptibility as compared to BHK and vero cells (Berting *et al.* 2010), no systematic investigation of their virus susceptibility has been published. However, Schuenadel *et al.* (2012) has reported susceptibility of CHO cells for cow pox virus. In light of earlier reports, present investigation was undertaken to study the prevalence of goatpox in surrounding areas of Durg district of Chhattisgarh state followed by adaptation of capripox virus isolates from goats in heterologous cells viz. CHO and CEF cells.

MATERIALS AND METHODS

Sample collection

An outbreak of goatpox was attended in five different villages of Durg district and surrounding areas of Chhattisgarh, India. Skin scabs were collected in glycerin-saline from 250 goats which showed clinical signs suggestive of pox i.e. fever, generalized papules or nodules, vesicles, pneumonia and death and preserved in refrigerator until screening for capripox virus. Scabs were ground into paste with pastel and mortar using previously sterilized fine glass powder and suspended in phosphate buffered saline (PBS) (pH 7.0) making a 10 % suspension. The suspension was put for three cycles of freezing and thawing and then the suspension was centrifuged for 15 min at 1500 rpm to remove large tissue particles and debris. Supernatant fluid was used as capripox virus antigen.

Reference capripox virus antigen and raising of hyperimmune sera

Sheeppox vaccine (BIO-MED private limited) containing attenuated virus was centrifuged for 15 min at 15000 rpm in the refrigerated centrifuge (Remi R_4C) and then filtered through millipore syringe filter (0.45µ). $10^{2.5}TCID_{50}$ titer of virus was used as antigen for preparation of hyperimmune sera. Hyperimmune sera were raised in rabbits as per protocol of Nour *et al.* (2012) with little modification. Serum was complement inactivated at 56 C for 30 min and stored in one ml aliquot at -20 C until used.

Identification of capripox virus antigen and determination of prevalence rate

Screening of capripox virus antigen in population was done by agar gel immuno diffusion (AGID) and countercurrent immune electrophoresis (CIE) test using hyperimmune sera raised in rabbits AGID was performed by taking 1% agarose in normal saline as per method described by Bhambani and Krishnamurty (1963). CIE was performed as per protocol described by Sharma *et al.* (1988). Electrophoresis was carried out by using a current of 8 mA per square cm area for 30 to 60 min Development of precipitation line was recorded as positive result. Failure to develop line for extended period of time (more than 72 hrs) was considered as negative result. Based on severity of clinical signs and laboratory confirmation by AGID; total prevalence rate, age wise prevalence and distribution of disease in various age group animals were determined (Table 1).

Isolation of capripox virus on chorioallantoic membrane (CAM)

Scab suspension was inoculated in to embryonated chicken eggs by CAM route as described by Cunningham (1966). The inoculated eggs, were incubated at 37ºC in horizontal position and candled daily up to five days and observed for embryo mortality. Virus was harvested

Table 1: Prevalence of goatpox in surrounding areas of Durg district of Chattisgarh

Particulars	Age group			Total
	Below 6 months	6 months to 2 years	2 years and above	
Number of animals in population	100	81	69	250
Number of animals affected	84	58	43	185
Age wise prevalence (%)**	84.00	71.60	62.32	74.00*
Distribution of disease (%)†	45.41	31.35	23.24	

*Total prevalence = Total number of animals affected / Total number of animals in population.

** Number of animals affected in particular age group /Number of animals in particular age group.

†Number of animals affected in particular age group/Total number of animals affected.

by collection of infected CAM after appropriate chilling to avoid bleeding. Virus isolates of CAM were passaged serially in embryonated chicken eggs three to six times. On each passage, CAM was harvested after five days of incubation and examined for characteristic pock lesions and further confirmed by AGID. Capripox virus antigen from CAM homogenate was prepared by making 10% suspension of infected CAM in PBS.

PREPARATION OF CEF AND CHO CELL MONOLAYERS

CEF cells were prepared from 9 to 10 day-old chicken embryo as per standard procedure (Cunningham, 1966). The CHO cells were procured (Hi Media Laboratories Limited, Bombay) and prepared as per manufacturer's instructions. Viability of CEF and CHO cells was checked using trypan blue stain. Cells at concentration of 10^5 cells per ml were dispensed in Eagle's minimum essential medium (EMEM) with Hank's salts and L-glutamine without sodium bicarbonate (Himedia laboratories) with 5% serum for growth. The uniform cell sheets were formed after 36 to 48 hr of incubation and were used for inoculation.

Virus inoculation

Capripox virus isolated from embryonated chicken egg (ECE) through CAM route was used for preparation of virus inoculums in PBS for adaptation in cell culture. The growth medium was removed and monolayer was washed once with EMEM. Capripox virus isolate @ 10^6 EID_{50}/ 0.1 ml was inoculated into 5 culture tubes, each tube received 0.2 ml of the inoculum. After 2 hrs of virus adsorption at 37ºC, excess inoculum from tubes was discarded and monolayer was washed with 2 ml of EMEM to remove unadsorbed virus. Maintenance medium containing 2% serum was then added at the rate of 2 ml to all cell culture tubes. Five uninoculated tubes were kept as controls. The tubes after inoculation were incubated at 37ºC and were examined daily for six days until maximum CPE was observed.

Confirmation of capripox virus isolates

Identity of CEF and CHO adapted capripox virus was confirmed by both AGID and CIE. Further, haemagglutinating property of capripox virus isolate was studied by haemagglutination (HA) test (Shakya *et al.* 2004) using 1% RBC suspension obtained from sheep, rabbit, dog and chicken blood and after 30 min of incubation at 28-30 C, HA titer was calculated.

RESULTS AND DISCUSSION

Prevalence of goatpox

The present study reported 74% prevalence of goatpox using AGID test. Likewise, earlier reports on outbreak of goatpox (Joshi *et al.* 1999) in Durg revealed 74.67 % morbidity and 48.21 % mortality. Distribution and prevalence of disease in young animals, a particularly in kid (below 6 month) was more as compared to those between 6 to 24 months and 24 months and above (Table 1). In agreement with present findings, Das and Pradhan (2006) also reported higher morbidity (69%) in kids. In contrast, Venkatesan *et al.* (2010) reported less morbidity and mortality but higher (60%) case fatality rate due to goatpox. Earlier report (Joshi *et al.* 1999) and present findings strongly suggest that goatpox in kids in local areas of Durg has emerged as virulent strain and thus strict prophylactic measures in kids and pregnant goats are essential.

Pock lesions produced by capripox virus on CAM

The isolate in ECE was identified as capripox virus on the basis of characteristic pock lesions in third passage.

Table 2: Morphological changes induced by capripox virus on CAM in serial passages

Level of passage	Morphology of the CAM
I	Oedema and thickening
II	Oedema, thickening along with haemorrhage and necrosis
III	Oedema, necrosis along with minute pock with average size of 0.5 mm in diameter. Most of these lesion were distributed on CAM over an area just below the artificial air sac
IV	Minute pock with average size of 0.7 mm in diameter
V	Minute pock ranging between 0.7 to 1 mm in diameter

Table 3: CPE induced by capripox virus in CHO cell culture in serial passages

Cultivation system	Passage level	Time required for CPE		
		Rounding and Clump formation	Increase no. of refractile cells	Complete detachment
CHO cell culture	III	72 hr	96 hr	120 hr
	IV	72 hr	96 hr	120 hr
	V	48 hr	72 hr	120 hr

In first and second passage CAM showed thickening with oedematous swelling along with haemorrhages and diffuse areas of necrosis (Fig.1 and 2). Virus induced changes in CAM during subsequent passage in embryonated eggs are shown in Table 2. Pock lesion observed on CAM during present study is in accordance with reports of Joshi (2000).

Propagation of capripox virus in CEF and CHO cell monolayers

On adaptation to susceptible cell culture, the appearance of characteristic cytopathic effects (CPE) is strongly indicative of virus growth. In the present investigation, there was no CPE in both CHO and CEF cells inoculated with capripox virus during first two passages. On the third passage, CPE was observed in CHO cell but in CEF cells, it did not produce any CPE even up to fifth passage (Table 3).

Fig 1: Capripox virus showing oedematous swelling along with haemorrhages on CAM

In contrast to present findings, Rao and Malik (1982) successfully adapted sheeppox virus in CEF cell culture. Capripox virus was detected positive following third passage in CHO and CEF cell by AGID as well as CIE tests. Detectable changes were observed as early as 72 hrs post-infection which consisted of rounding of cells with cytoplasmic vacuolation. Subsequently at 96 hrs, few cells detached from the monolayer as evident by increase in number of retractile bodies. Rounding and clumping of cells was more marked with separation of cells from sheet (Fig. 1 and 2). At the end of 120 hrs, further degeneration of cell sheet was observed and most of the cells appeared floating in the medium. Likewise, similar CPE was observed in vero cells by capripox virus (Raof *et al.* 2008). Growth of capripox virus in CHO cell may correspond to its fibroblastic nature (Golenbock *et al.* 1993).

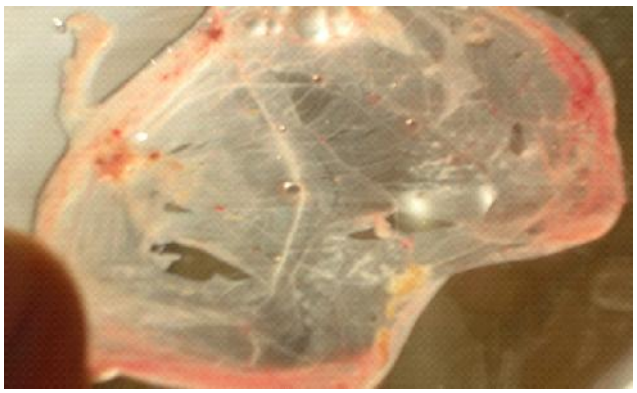

Fig. 2. Capripox virus showing minute pock lesions on CAM

There are not any earlier reports on adaptation of capripox virus in CHO cell, however Schuenadel *et al.* (2012) cultivated cow pox virus (a member of family Poxviridae) in CHO cell and observed that CHO cell is permissive for cow pox virus; can effectively multiply and produce characteristic CPE in CHO cells. Whereas, Ramsey-Ewin and Moss (1998) reported that CHO cells are readily killed by vaccinia virus within 2-3 days. CHO cells offer the advantages that they are easily genetically manipulated, can be adapted for large-scale suspension culture and can grow in serum-free and chemically defined media which ensures reproducibility between different batches of cell culture (Lai *et al.* 2013). Above facts encouraged to use CHO cells in present investigation.

CHO and CEF cell adpated capripox virus isolates at passage level I, II and III failed to agglutinate erythrocyte of all species tested. However, CHO adapted isolates of 4th and 5th fifth passage agglutinated only chicken erythrocyte and yielded HA titer of 1:256, which is in agreement with observation of Raof *et al.* (2005). However in a report by Shakya *et al*. (2004), goatpox virus failed to agglutinate the sheep, goat, pig, rabbit, dog, chicken and human type 'O' erythrocytes.

After formation of Chhattisgarh state in 2000, no new outbreaks were officially reported. However, present study reveals that the severity and extent of goatpox has increased and warrants immediate attention, particularly in the events of unknown history about vaccination against sheep and goatpox. Considering the severity of clinical symptoms, morbidity and distribution (particularly in kids) in current outbreaks, there is strong possibility about re-emergence of the disease in adjacent areas. This study offers a traditional, convenient and preliminary approach for diagnosis of capripox virus infection and help to formulate strategies on the basis of local field isolate of capripox virus modified by adapting it to suitable host system. Capripox virus isolate from goat was successfully adapted and propagated in CHO cell culture. Further studies would be required to investigate the infectivity titre of virus in CHO cell and to characterize capripox virus so as to develop diagnostics and candidate vaccine.

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