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COMPARATIVE SENSITIVITY OF PCR AND CELL CULTURE TECHNIQUE FOR THE IDENTIFICATION OF GOATPOX VIRUS

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ABSTRACT

Goat pox is the most serious of the pox diseases of livestock and can cause heavy production losses. The study was designed to compare the sensitivity of PCR analysis with the cell culture technique for detection of goat pox virus. For this purpose a total of one hundred skin and scab samples were collected from cattle market, hide market and slaughterhouse in district Lahore Pakistan.. Vero cell line culture was used for the isolation of virus. For PCR analysis, the sequenced genome of KS-1 strain primer of Capri poxvirus was used. Out of one hundred, 76 samples were positive by PCR while 46 were positive by cell culture technique. It was found that cell culture gives 17%, 9% and 20% positive results while PCR give 22%, 21% and 33% positive results in Cattle markets, hide markets and tanneries and slaughter houses, respectively. It was concluded that that PCR is more specific and sensitive technique as compared to cell culture method for detection of goat pox virus.

Key words: Goat pox virus, Vero cell line, PCR, cell culture.

INTRODUCTION

The family Poxviridae is comprised of sheep pox virus (SPV), goatpox virus (GPV), and lumpy skin disease virus of sheep, goat and cattle, respectively. Goatpox is the most serious of the pox diseases of livestock and can cause heavy production losses. The economic losses occur in the form of mortality, reduced productivity and lower quality of wool and leather Parthiban *et al.,* (2005). The disease is a serious threat to the goat farming community in endemic areas and is associated with trade restrictions following outbreaks. Capripox virus causes a severe and highly contagious disease in goats which is listed in Group 'A' diseases of the OIE (Carn, 1993).

Capripox virus causes goatpox disease, which is characterized by high morbidity and mortality. The disease is prevalent in both the rainy and winter seasons, but the mortality is high in rainy season due to humid environment, which is conducive to the rapid replication of the virus (Khan, 2005). The incubation period is about 5-14 days. The transmission of virus mainly occurs through direct contact via the aerosol, but indirect contact and mechanical transmission by insects can also occur. The affected sheep and goats shed the virus at every stage of the disease. The virus persists for at least three months in the wool, hair and scabs of infected animals. It can survive for up to six months in the environment and can be readily spread by fomites. In endemic areas spread occurs mainly in summer Bhanuprakash *et al.* (2006).

Typical pox lesions appear on the skin and on the respiratory and gastrointestinal mucosa. Skin lesions erupt in one to two days. The lesions extend over all part of the skin but are most obvious in hairless areas, such as on the face, ears, maxillae, groin, perineum and under the tail. Lesions may be seen on the mucous membranes of the mouth, nostrils and vulva. The lesions follow the classical pox cycle of skin erythema, papule (0.5–1.5 cm diameter), vesicle, and pustule with exudation, encrustation and scab formation, over about two weeks. Matting of the fleece occurs due to the exudates from ruptured pustules. Healing of skin lesions is slow, taking five to six weeks. Deaths may occur at any stage of the disease with peak mortality occurring about two weeks after the appearance of lesions Gulbahar *et al.,* (2000).

The diagnosis of the disease is based on gross lesions, clinical signs, virus isolation, and serology. But all these diagnostic procedures have limited value in accurate diagnosis of the disease in one way or another for example gross clinical findings can easily be confused with disease like contagious ecthyma and contagious pustular dermatitis. Similarly the isolation and serological techniques have limited specificity and accuracy due to antigenic and biochemical similarities of causative agents. These methods are also time consuming, laborious, and require sterile conditions (Lefevre, 1983, Kitching and Carn, 1996). Therefore, its control and eradication constitute a never ending challenge. But unfortunately the published scientific work on *Goatpox* infection is very scanty Elzain *et al.* (2003). The present study was designed to identify the virus by both

conventional and advanced molecular techniques and to compare their specificity and sensitivity.

MATERIALS AND METHODS

Samples: A total of 100 skin and scabs samples from clinically positive cases were collected from goat markets, Government slaughter houses and hide markets of Lahore, Pakistan. The samples were preserved for isolation of virus on Vero cell line and its identification was made through PCR.

Virus isolation: All hundred samples consisting of 45 skins and 55 scabs samples were triturated and homogenized in Dulbeco Modified Essential Medium (DMEM) with antibiotics making up a 50% (w/v) solution. Suspensions (50%) of the samples were made in Dulbecco's Modified Essential Medium (DMEM) containing penicillin 1000IU/ml, streptomycin 1000µg/ml, amphotericin B 25mg/ml and kanamycin 500µg/ml. The homogenates were centrifuged at 1,500 rpm for 15 min. The supernatant was collected, filtered through 0.45 µm membrane filters and stored at -80^{o}C until use. Monolayers of Vero cells grown in 25 cm^2 tissue culture flasks were inoculated with sample supernatant using two flasks per sample. Following incubation at 37°C for 2 hours the inocula were discarded, the flasks were washed three times in Dulbecco MEM, followed by the addition of maintenance medium containing 5% fetal calf serum (FCS), penicillin 10,000 UI/ml, streptomycin 100 µg/ml, kanamycin 50 µg/ ml and amphotericin B 2·5 µg/ml. The field isolated Capripoxvirus was inoculated on Vero cell lines according to the method of Elzein *et al*., (2003) and Balinsky *et al*., (2008). The flasks were incubated at 37°C and were observed daily the appearance of cytopathic effects for up to 14 days post-inoculation. Two more blind passages were carried out for samples that were initially negative for cytopathic effects (CPE). When 100% CPE was observed, the flasks were frozen at -20 C (after pH adjustment). The virus was harvested after two freeze-thaw cycles.

Polymerase chain reaction (PCR): For PCR analysis the standard protocol was followed with little modifications as described by Mangana-Vougiouka *et al.,* (1999)

Primers:	OLIGO KS- 1. 5: KS 1. 6 primers was used
Left primer	GTG TGA CTT TCC TGC CGA AT
Right primer	TCT ATT TTA TTT CGT ATA TC

DNA extraction: DNA was extracted from the field isolates by using DNA Purification Kit 'Fermentas life sciences Pure Extreme ^{TM}, EU' according to the manufacturer's instruction with some modifications as follows. Uninfected Vero cells and a vaccine strain of goatpox (obtained from the Veterinary Research Institute, Lahore Pakistan) were used as negative and positive controls, respectively. Briefly, the cell culture cells, triturated scabs and tissue samples were centrifuged at 13,000 rpm for 15 min, the supernatant was discarded and pellet was re-suspended in 200µl of TE buffer. 200µl of re-suspended cells was taken in 1.5 ml Eppendorf tubes separately and mixed by vortex for few seconds. When the pellet was completely re-suspended, 400 µl of cell lyses solution was added and homogenized. Eppendorf Tubes were incubated at 65 ^{o}C for 30 minutes, in thermostatically controlled water bath. Immediately 600 µl of chloroform was added and gently emulsified by several inversions. Spinning was done at 13,000 rpm for 10 minutes at 4^{o}C. The aqueous phase of supernatant containing DNA was carefully collected in new Eppendorf tubes. 800 µl of precipitation solution (720 µl of sterile de-ionized water and 80 µl of supplied 10x concentrated solution) were added in each tube. The tubes were vortexed followed by centrifugation at 13,000 rpm for 10 minutes at 4^{o}C. Supernatant was removed carefully and dissolved the DNA pellet in 100 µl of 1.2 % NaCl solution. 300µl of cold absolute ethanol was added and the DNA was allowed for few minutes to precipitate at -20^{o}C. Then by centrifugation at 13000rpm for 10 minutes the DNA pellet was obtained and ethanol as supernatant was discarded. Then DNA pellet was washed with 70% cold ethanol and finally dissolved in sterile de-ionized water 50 µl. All the components of kit were stored at room temperature to maintain the stability of the contents according to the manufacturer's instructions.

A total of 5 µl sample of each Purified genomic DNA isolated from goatpox virus infected Vero cells, scabs and tissue samples were subjected to PCR in 25 µl of the final volume of a 10x reaction mixture containing, 2.5 µl of 10X PCR buffer, 2.5 µl of $MgCl_2$, 2.5ul of 2.5 mM dNTPs, 0.3ul of Taq DNA polymerase of 5U/ ul, 1ul of forward primer, 1ul of reverse primer, 5 µl of DNA sample and 10.2ul of distilled water were used for each reaction. The PCR reaction mixture was transferred to tubes of a thermal cycler. The cyclic parameters were: 94ºC for 4 min followed by 35 cycles of de-naturation at 95ºC for 10 sec, annealing at 55ºC for 10 sec and extension at 72ºC for 10 sec and a final extension of 72ºC for 15 min. The amplified DNA fragments were analyzed by agarose gel electrophoresis.

RESULTS

Goatpox virus was isolated from 46 samples in Vero cell cultures. The CPE began at 24hrs after virus inoculation and reached a maximum after 96-120 hrs. The titer of the virus at was $10^{6.3}$7 days post infection. The CPE consisted of rounding tract formation, retraction and ballooning, nuclear vaculation, chromatin formation, loss of continuity of the monolayer and the appearance of

basophilic and eosinophilic intracytoplasmic inclusion bodies. Non specific cytopathic effects, diffused areas of rounded cells with out plaque formation, and non detachment of the cell layer were also noticed. One week after inoculation, destruction of the monolayer was evident. Passage one in Vero cell was stored at -80^{o}C. Uninfected Vero cell monolayer and goat pox-induced CPE are shown in Fig. 2. Cell culture gave 68%, 25.71% and 50% positive result in Cattle markets, hide markets and tanneries and slaughter houses, respectively.

A total of 100 scabs and skin biopsies were examined by PCR; 76 were positive. Out of 40 samples from slaughter houses 18 scabs and 15 tissues sample were positive through PCR. Out of 25 samples collected from cattle markets consisting of 20 scabs and 5 skin tissues, 17 scabs and 5 skin tissues were positive. Similarly a total of 35 samples consisting of 15 scabs and 20 skin tissues were collected from hide markets and tanneries. Out of these 7 scabs and 14 skins tissue samples were found positive. In this way the total PCR result for scabs and skin tissues from all areas were 76.36% and 75.56%, respectively. This primer offered sensitive and specific detection of 149 bp product for Goat pox infection. The specific 149 bp PCR products were visualized on agarose gel electrophoresis (Fig. 3).

The results of both cell culture and PCR were compared and were found that cell culture gave 68%, 25.71% and 50% positive results while PCR gave 92%, 60% and 82.5% positive results in Cattle markets, hide markets and tanneries and slaughter houses, respectively. The PCR produced more positive results than the cell culture technique (Fig. 1).

DISCUSSION

Goat pox being an important disease in Pakistan, it is essential to accurately diagnose it in order to sort out ways and means for rapid and timely control of this fatal disease to minimize the economic losses. A tentative diagnosis of pox infection is made on the basis of clinical signs symptoms but an accurate and reliable diagnostic technique is required to confirm the disease. Little work has been conducted on the diagnosis of pox infection in Pakistan. However, workers in other countries of the world have reported advanced and sensitive methods for pox virus diagnosis in goats. The disease must be differentiated from contagious echthyma, bluetongue, myotic dermatitis, sheep scab mange, caseous lymphadenitis, photosensitization Peste Des Petits Ruminants, pustular dermatitis, and other exanthema conditions.

Serological tests are used in the diagnosis of goat pox infection. Various serological tests, such as virus neutralization test, (OIE, 2000), ELISA (Heine *et al*; 1999), indirect ELISA (Carn *et al*; 1986), immunofluorescence, agar gel immunoprecipitation test assay, viral neutralization test, (Managana-vuogiouka *et al*., (2000), indirect fluorescent antibody test, are used for the diagnosis of Goatpox virus. But these tests are not very sensitive and sometimes give false positive results due to cross reactivity.

The CPE begins at 24hrs after virus inoculation which reached to maximum after 96-120hrs having a titer of $10^{6.3}$ on the 7th day. The same was reported by Eltayb *et al., (*2003). The CPE were consists of rounding tract formation, retraction and ballooning, nuclear vacuolation, chromatin formation, loss of continuity of the monolayer and the appearance of basophilic and esinophilic intracytoplasmic inclusion bodies. The same findings are in conformity with Hossamani *et al.,* (2008). The percentage of isolation of virus from skin samples was more as compared to the scabs samples which is due to the fact that skin samples are consisting of relatively more fresh tissues providing suitable environment for the survival of virus. While the scabs are hard and desiccate in nature where there are limited chances of survival of virus.

The virus isolation was more from slaughter houses and cattle market as compared to hide market and tanneries. This is due to the fact that the samples in cattle market and slaughter houses were collected from live animals where the tissues were fresh as compared to the samples collected from hide market where the tissues were dead and dried providing non conducive environment for virus replication. The 2nd part of the experiment was to develop a rapid, sensitive and reliable technique for confirmation of the growth of Goatpox virus on Vero cell line. Confirmation is necessary because goat pox is often confused with many infections and its differentiation is usually not easy through conventional techniques. Due to its slow growth and the requirement of additional passages in the virus isolation process, it is difficult to isolate viruses in the Capripoxvirus genus, but at 14-15th passage it grows easily (Mangana-Vougiouka, 1999). The PCR is generally a sensitive and reliable technique for confirmation of virus in cell culture and body tissues. In the present study, the protocols and primer of Gershon and Black (1989) and Gershon *et al.,* (1989) were used for identification of Goatpox virus in the culture. This primer offered good detection of 149 bp product for Capri pox infection. The same findings were also reported by Mangana-vougiouka *et al.,* (1998), Heine *et al* (1999) and Parthiban *et al.,* (2005). During our study, the results of cell culture and PCR were compared, and cell culture gave 68%, 25.71% and 50% positive results while PCR give 92%, 60% and 82.5% positive results in cattle markets, hide markets and tanneries, and slaughterhouses, respectively. The PCR produced more positives than cell culture technique. The selected primer has successfully amplified the target DNA.

During the study the result of both cell culture and PCR were compared and were found that cell culture give 68%, 25.71% and 50% positive results while PCR give 92%, 60% and 82.5% positive results in cattle markets, hide markets and slaughter houses, respectively. Goatpox virus isolation is difficult, it grows slowly and requires additional passages, but when passaged 14-15 times in these cells it grows easily. The PCR is more sensitive and reliable technique for confirmation of virus in cell culture and body tissues. The findings of present study are justified by the observations of Heine *et al*., (1999) and Parthiban *et al.,* (2005). The PCR was more sensitive than cell culture technique. From the findings of this study it can be concluded that the cell culture method is less sensitive, time consuming and laborious as compare to PCR analysis which is more sensitive and rapid technique for accurate diagnosis of goat pox virus.

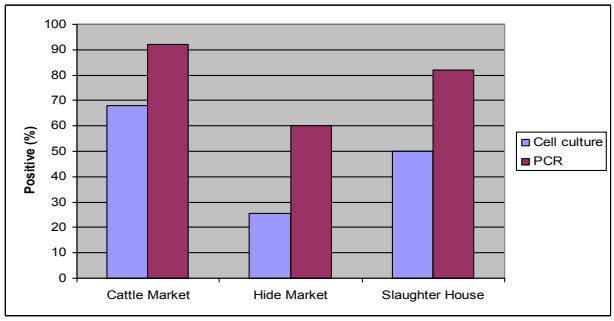

Figure 1. Comparison of PCR and cell culture for the detection of goatpox virus in different source of samples

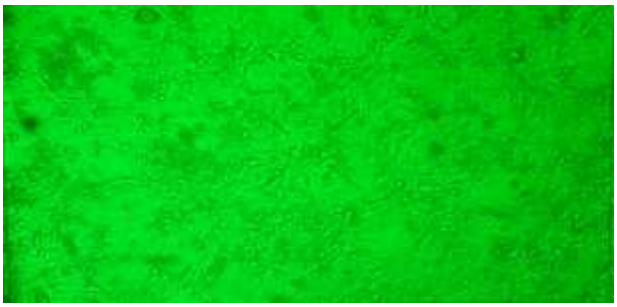

Figure 2: Cells become round, aggregate culminating in syncytia formation after infection.

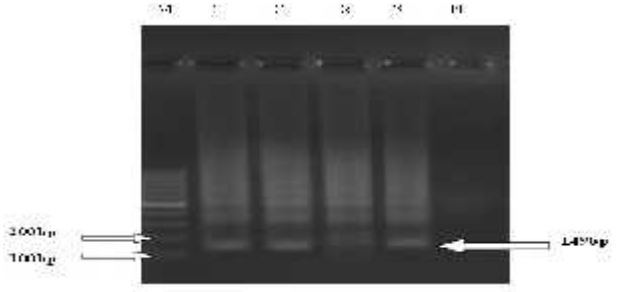

Fig. 3. DNA amplification of *goatpox virus* showing an amplicon size of 149 bp by electrophoresis.

M = 100bp ladder, C =positive control, N =negative control 1, 2, 3 =sample

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