



PREVALENCE AND PCR BASED MOLECULAR CHARACTERIZATION OF GOAT POX VIRUS FROM FIELD OUTBREAKS OF MULTAN AND BAHAWALNAGAR, PAKISTAN

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Abstract

This study was designed to check the prevalence and PCR-based molecular characterization of goat pox virus (GTPV) in the Multan and Bahawalnagar regions of Punjab, Pakistan. Capripox virus (CPPV) is the cause of goat pox (GTP) and sheep pox (SPP) disease, it highly affects the morbidity and mortality rate of goats and sheep. In this study, the 80 tissues and blood samples of goats were collected on age basis from the goat farms, slaughter houses, tanneries and domestic animals. The epidemiological data was also collected. The collected samples were processed for DNA extraction. We characterized the goat pox virus (GTPV) with specific primers of P32 gene by PCR. Then each amplified product was analyzed by agarose gel electrophoresis visualized by UV fluorescence light. This study showed that Infants of goats (2-10 months) in Multan showed 25% while adult goat in Multan showed 14.2 % positive results. In Bahawalnagar, the affected infants of goats (2-10 months) found were 31.25% while adult infected goats were 11.1%. Both primers were equally effective for the characterization of unknown samples. The most effected goats were adult female and infants.

Key words: CPPV, GTPV, GTP, SPP, SPPV, P32

Introduction

Domesticated animal play significant roles in the economy of Pakistan and contribute around 56% in development agribusiness and around 11% to the GDP. Animals incorporate the cows, wild oxen, sheep and goat (Rehman et al. 2017). The flexibility of goats to various conditions and flawless association results to extend their world, under which those animals create and spare themselves. Pakistan is third most prominent country with relevancy glut of goats after China and India and having 66.6 million heads. While Pakistan is at fourth position in goat's gutter and sheep growing international locations with each year generation of 822 thousand tons of drain and 657 thousand tons of meat (Aziz 2010). Disease predominance is affected by organic framework, physiographic, soil sorts, precipitation, temperature and relative moistness (Bhanuprakash et al. 2006). SPP and GTP virus might be inactivated at 65°C within 30 minutes or 56°C within 2 h. They can make infection at a pH in range of 8.6 and 6.6. These viruses are inclined to particularly acidic or basic pH; 2% HCl can crush these viruses within 15 min (Yeruham et al. 2007, Yune and Abdela 2017).

Animals are helpless all through all age associations, and it has an exceptionally vital part in agrarian monetary framework. From the field of announced ailment flare-ups, it was learnt that the ailment could not be seen in immunized papules however saw in unvaccinated animals (Roy et al. 2018).GTP infection is

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transmitted by contact with infected animals, their vaporizers, nasal release, salivation or dried scabs. The infections are also promptly transported on garments. Some insects e.g. the stable fly (*Stomoxys calcitrans*) act occasionally as mechanical vectors. Epidemiology of the ailment is likewise essential in diagnoses of SPP and GTP. Developing life clearing and auxiliary pneumonia are also viewed on the complexities (Juneja and Ganguly 2017, Yune and Abdela 2017). Movements of infected animals act as principle source of spreading infections. SPPV and GTPV may make critical harm fleece and covers up, diminished lamb and drain creation (Babiuk et al. 2008). The death rate of sheep and goat will currently and again be significantly high, among sheep and grown-ups (Tuppurainen et al. 2017). CPPV cause high mortality and morbidity rate in dairy cattle, goats and sheep (Sajid et al. 2013). The disease is basic both in stormy and winter season; however, the death rate is highest in the stormy season due to the rapid transmission of virus. It was also likewise watched that 33.33% disease was in roaming groups and 10% in settled crowds (Massoud et al. 2016). As of late landowners of Tharparkar and its encompassing zones have endured immense financial misfortunes as death of thousands of sheep because of this disease. Unstable and specific nuclear procedures are used for acknowledgment of CPPV concentrating on the RPO30, P32 and GPCR characteristics (Zhou et al. 2012). It is understood that heterogonous diagnostic reagents tend to be less productive than homologous reagents for corroborative finding (Yune and Abdela 2017). Prognosis of SPP is often supported extraordinarily function clinical symptoms ELISA check virus neutralization test, virus isolation (Tian et al. 2010) and PCR measures (Balinsky et al. 2008, Adedeji et al. 2019).

In previous studies the seroepidemiology data of goats were studied only in district Layyah Punjab Pakistan (Masoud et al. 2016). In another study the prevalence was checked in the areas of Punjab Pakistan and its needed to further plan the study to control and eradicate this disease by control measure and by proper vaccination (Sajid et al. 2012). In further study we should do cloning and should find the way to control this disease in goats. The objective of this study is to check the prevalence of Goat pox disease in Multan and Bahawalnagar regions and molecular characterization of goat pox virus by PCR. The PCR test has enormous potential for the discovery of pathogens and for the most part utilized as the touchy and corroborative determination of the personality of the illness. The utilization of PCR strategy for SPP and GTP infection distinguishing proof thought to be a basic, quick and indicative technique.

Materials and Methods

Ethical approval

As per the committee for control and supervision of experiments on animal's guidelines, studies involving the collection of field clinical samples do not require any approval from the Institute's Ethics Committee. Study areas and sample collection. The present study was designed to collect the samples of infected goats based on age and characterize them by PCR from Multan and Bahawalnagar regions of Punjab, Pakistan. The samples were collected from different hides, slaughterhouses and goat markets from Multan and Bahawalnagar regions. It comprises five administrative units, Minchan Abad, Chishtian, Fort Abbas, Haroon Abad, and Bahawalnagar.

Blood samples, hair samples, and skin tissues were collected from the animals showing typical signs and symptoms of goat pox disease. About 80 samples were collected from infected goats present in different herds. Blood samples were collected in EDTA tubes and tissue samples were collected in SDS solution without EDTA tubes. By following Biosafety procedures all the samples were stored in the icebox and brought to GCUF Zoology Research Laboratory through proper transportation and packaging. The collected samples were centrifuged on 10000 rpm for just one minute so, the debris was settled down and a clear solution was used for the further process. Viral DNA was isolated from collected samples by following the procedure of (Sambrook et al. 1989).

The DNA extraction method

Fifty μ l from each collected sample was taken in Eppendorf tubes and homogenization was carried out by using 400 μ l TAE buffer. Then added 400 μ l of 20% SDS and 100 μ l of 20 mg/ml proteinase K. After Incubation, for 4 hours at 55°C these tubes were vortexed for 15-30 sec. The supernatant was taken after 10 min centrifugation and transferred to another tube. DNA was precipitated by adding 300-400 μ l of isopropanol or super cold 100% ethanol and kept at -4°C for 60 minutes. Centrifuged again for 10 minutes. Pellet was washed with 70% ethanol and was dried for 10-15 minutes. DNA was permitted to suspend in 25 μ l of sterile water (D_3H_2O) for further processing. Nanodrop was used for the DNA quantification (Bowden et al. 2008).

Primers

A conserved region of *P32* gene was used for the detection of GTPV. The following table shows the sequence and nucleotide position of the reported primer used in this study (Bowden et al. 2008) (Table 1).

Table 1. Reported and designed primers of goat pox virus

| General primers of goat pox | Sequence (5'-3') | PCR product (bp) | Reference |
|-----------------------------|--------------------------------|------------------|----------------------|
| (P1) Forward | 5'-CTAAAATTAGAGACTATACTTCTT-3' | | |
| (P2) Reverse | 5'-CGATTTCATAAACTAAAGTG-3' | 969 bp | Bowden et al. (2008) |

PCR conditions and reactions

For PCR of random amplified Polymorphic DNA analysis, the concentration of genomic DNA 10 \times PCR buffer with $(NH_4)_2SO_4$, $MgCl_2$, dNTPs, 10-mer random primer and DNA *Taq* Polymerase were optimized. The 10 base oligonucleotide primers used for amplification of genomic DNA were obtained from Gene link Company. PCR was conducted in 50 μ l PCR tubes containing the 25 μ l master mixture. PCR reaction was done in Persona Autorisierter Master cycler of the Eppendorf, Germany. The PCR for goat pox virus were performed by using 10 μ l of template placed in 50 μ l of the ultimate extend of a 10 X reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 mM of dNTP and 100 pmol of primer sets for the gene encoding for the viral attachment protein as described by (Ireland and Binopal 1998) and 2U *Taq*-DNA polymerase. Amplification consisted of an initial denaturation step at 94°C for 4 min, which will be followed by 35 cycles at 47°C for 1 min, 72°C for 1 min, 95°C for 45 sec and, finally, extension at 72°C for 10 min in a thermal cycler.

PCR products were visualized in 1% agarose gel containing ethidium bromide. PCR products were evaluated on 1.5% agarose gel prepared in TAE buffer. DNA samples were then loaded with DNA loading buffer. The gel was run out at 120 volts for about 30-40 minutes. The gel has been examined under ultraviolet Transilluminator and photographed using a gel documentation system (WEALTEC, Dolphin-Doc) (Chopade et al. 2013).

Results

The clinical signs of GTP were found in the form of pustules, papules, nodules, and scabs mostly on the hairless areas such as groin and perineum in addition to nose, eyes and lips making the feed intake painful. It was found that overall prevalence of CPPV was more in Bahawalnagar, as we found the affected animal which shows the typical signs have prevalence of 24% and in Multan 20%. This above percentage showed the combined effect of the CPPV on sheep and goat. While the separate effect shows some differences regarding the percentage. Infants of goats in Multan showed 25% positive results while adult goat in Multan showed 14.2% positive results. In Bahawalnagar, the affected infants of goats found are 31.25% while adult goats with positive signs are 11.1% (Table 2).

Table 2. PCR based detection of virus from suspected samples collected from different herds and Slaughter houses

| Species | Multan | | | % Positive | Bahawalnagar | | | |
|----------------------------|-----------|----------|---------|------------|--------------|----------|---------|------------|
| | Samples | | | | Samples | | | % Positive |
| Infant goats (2-10 months) | 16 | Infected | Healthy | 25% | 32 | Infected | Healthy | 31.25% |
| | | 4 | 12 | | | 10 | 22 | |
| Adult goat | 14 | 2 | 12 | 14.2% | 18 | 2 | 16 | 11.1% |
| Total | 30 | | | 20% | 50 | | | 24% |

The PCR measure was observed delicate and the exactness and unwavering quality was affirmed by sequencing the relating amplicons of GTPV field disengage accessible for this examination (Fig. 3 and 4). The flow thinks about recommended that the two preliminaries of P32 and other quality can be utilized for atomic recognizable proof of GTP malady. The accessibility of a simple to-utilize sub-atomic technique is required for the distinguishing proof of CPPV. The distinguishing proof of appropriate focus in the viral genome of P32 to separate antibody strains from GTPV field disengages incredibly decreases the expenses, by permitting the sequencing of little genome pieces. The present test is expected to be utilized by every veterinary research facility, incorporating those with constrained assets. It can also be utilized as a lead instrument for the immediate screening of neurotic examples gathered in CPPV flare-ups, particularly those happening in already immunized little ruminant populaces. (Fig. 1 and 2).

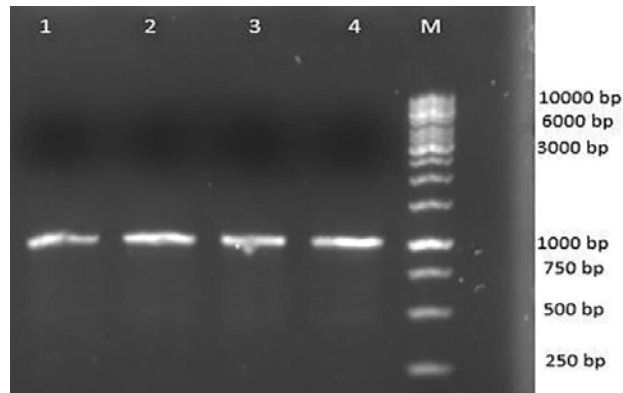


Fig. 1. Agarose gel showing 969 bp PCR product of p32 gene of Capri pox virus using P1 and P2 primers (Lane 1- 4: positive samples, Lane M: 1Kb ladder)

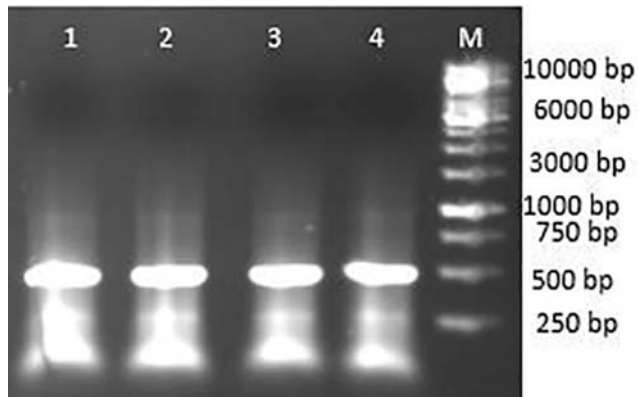


Fig. 2. Agarose gel showing 530 bp PCR product of p32 gene of Capri pox virus using S1 and S2 primers (Lane 1- 4: positive samples, Lane M: 1Kb ladder)

Discussion

Goat Farming is incredible and popular business model in Pakistan and about 66.6 million goat population recorded per economic survey of 2016. CPPV contamination of sheep and goat effects in most important monetary losses and it also hinders the worldwide trade (Anonymous). In Pakistan, the disease is primary in arid regions accompanied by southern and northern regions of Punjab Pakistan (Sajid et al. 2012).

The current study of CPPV based on the prevalence and characterization of SPPV and GTPV in the areas of Pakistan. SPPV and GTPV are endemic in some countries. These viruses can easily be diagnosed clinically as SPPV and GTPV, but it cannot differentiate at field level. Although, there are many reports of CPPV outbreak and prevalence in goats and sheep and their causative agents. But there is a need to identify causative agents properly in outbreaks of CPPV. There are some game animals play role in maintaining and

cause an outbreak of CPPV for the identification and differentiation of GTPV, SPPV and LSDV, the phylogenetic analysis used in many studies. CPPV, RPO30, PCR used for the fast differentiation of GTPV and SPPV without using the technique of gene sequencing. Mahmoud and Khafgi (2016) used the species-specific primer, CPPV RPO30 PCR method to detect and identify the infection. Their results declared that the sequencing RPO30 gene-based PCR assay give a good picture of molecular epidemiology of CPPV infection.

In Previous study P32 primer was used for the detection of GTPV by PCR method Bora et al. (2018). P32 primer was also used for differentiation of GTPV, SPPV, and LSDV by PCR method (Shehbaz and Hussain 2017). It was also found that the PCR assay developed a specific and sensitive method for differentiation and detection of infection in SPPV and GTPV (Zhou et al. 2012). It was declared that the PCR and EM are a rapid and sensitive method for differentiation and characterization or identification of SPPV and GTPV (Abd-Elfatah et al. 2018, Adedeji et al. 2019). As many other studies concluded that the P32 primer and PCR methods are the best parameters used ever for the phylogenetic analysis and use of RPO30 gene-based PCR assay used for molecular epidemiology (Yan et al. 2012, Santhamani et al. 2013, Venkatesan et al. 2010, 2014 & 2016, Mahmoud and Khafgi 2016, Zhao et al. 2014 & 2017, Karapinar et al. 2017, Roy et al. 2018).

It was found that the clinical signs of GTP may be variable. Skin lesions are seen and characterized by papules, nodules, pustules, and scabs. Overall prevalence of CPPV was more in Bahawalnagar as we found the affected animals which have prevalence of 24% and in Multan 20%. This above percentage showed the combined effect of CPPV on adult goat and infant goat. While the separate effect shows some differences regarding the percentage. Infant Goats in Multan showed 25% positive results while adult goats in Multan showed 14.2% positive results. In Bahawalnagar, the affected infant goats found are 31.25% while adult goat with positive signs is 11.1%. The samples were characterized first by reported primers. P1 and P2 primer pair yield a PCR product of 969 bp representing the *P32* gene. Then newly designed primer was also checked and found that these primers are also giving good results, while new designed S1 and S2 primer yielded a PCR product of 530 bp. Disease caused by CPPV is important causes of economic loss in goat and sheep farming. It was determined that 33.33% disease was in nomadic herds and 10% in settled herds (Masoud et al. 2016). Farmers of Tharparkar and its surrounding areas have suffered huge economic losses in the form of death of thousands of sheep due to this disease.

Based on the results of the present study following conclusion are drawn: *P32* gene is an important gene used for characterization of GTPV. Specific primers are used which gives 969 bp product of PCR. Also, the newly designed primer has given 530 bp product of PCR. Both reported, and newly designed primers showed positive results for identification of GTPV and negative as normal skin samples. The seasonal calendar of GTPV was outlined by informant groups and it was claimed to occur during the long and short rainy seasons of a year. Furthermore, GTP was listed to be one of the most common five goat diseases in the area by the farmers. Generally, the disease and associated morbidity and mortality were less commonly seen in adult age groups as compared to young age groups. *P32* is the major immune dominant gene having amplicon size of 1024 bp and 1027 bp in GTPV and SPPV respectively. Also, *P32* is gene sequencing data widely used for differentiating SPPV and GTPV and phylogenetic analysis of CPPV (Zhou et al. 2012). Several researchers used the *P32* gene to detect SPV and GPV because it contains a most significant antigenic determinant present in all species of CPPV genus (Tian et al. 2010). Based on *P32* gene analysis,

it used to be determined the GTPV sequences are all most conserved. From the field of stated disorder outbreaks, it was once learned that the disease may want to no longer be determined in vaccinated populations but observed in unvaccinated animals (Roy et al. 2018).

Conclusion

The results of this study showed that disease mostly prevailed in Bahawalnagar (24%) than Multan (20%). We declare this % age by using gene sequencing of P32. The animals were declared positive based on the PCR test. The normal skin samples were also preceded for negative control, but the virus was not detected, and vaccination of blood samples was taken as positive control. PCR was optimized for detection of Capri pox virus using specific primers. The samples were characterized first by reported primers.P1 and P2 primer pair yield a PCR product of 969 bp representing the P32 gene. Then newly designed primer was also checked and found that these primers are also giving good results, while new designed S1and S2 primer yielded a PCR product of 530 bp. After conducting the study, we reach a point that the major factor in the spread of the disease was a movement of nomadic peoples along with their animals in different areas. It can be controlled by the carpet vaccination at the entry point and damping down vaccination in the endemic areas. Similarly, a bivalent vaccine of Capripox can give good results.

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