Indian Journal of Animal Sciences **90** (2): 152–155, February 2020/Article

Tetra-primer amplification refractory mutation system-polymerase chain reaction (TARMS-PCR) assay in genotyping of single nucleotide polymorphism in goatpox virus *p*32 gene

ADARSH MISHRA[1] and PARIMAL ROY[2]

Central University Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 051 India

Received: 25 April 2019; Accepted: 10 July 2019

ABSTRACT

Single nucleotide polymorphisms (SNPs) are most often associated with some pathological implications. Screening out the presence of such mutations is extremely sought to know the nature of the disease outbreak. Furthermore, the allele specific distributions of the virus are to be known for effective epidemiological strategies. Tetra-primer amplification refractory mutation system-polymerase chain reaction (TARMS-PCR) is a simple, rapid and inexpensive technique as compared to high thoroughput sequencing methods for genotyping SNPs. In the present report, a novel TARMS-PCR was utilized to ascertain the presence of a particular allele ($_{645}GTPV^{C/T}$) in the *p32* gene of goatpox virus (GTPV), one of the most widespread *Capripoxvirus* affecting small ruminants exhibiting moderate to even severe pathological consequences in the endemic areas. It was found that GTPV of Chinese origin are $GTPV^{C/T}$ type whereas only single genotype ($GTPV^{T}$) was found among GTPV of Indian origins. Possibly, this is the first report of development of a TARMS-PCR technique for genotyping of virus to ascertain the presence of a specific allele. This technique can be applied further to unveil the presence of deleterious mutations in any other viral genome. Further, this technique can be applied for cross-border surveillance of GTPV among China and India.

Key words: Genotyping, Goatpox virus (GTPV), *p*32 gene, SNPs, Tetra-primer ARMS-PCR

Goatpox is one of the most endemic *Capripox virus* diseases affecting small ruminants in central and North Africa and Asiatic countries especially India and China (OIE, 2010). It affects goats most commonly, though some strains may affect sheep (Bhanuprakash *et al.* 2010; Zhou *et al.* 2012). The disease is characterized by rise in body temperature at initial stage followed by development of papular lesions in the skin, formation of vesicles, pustules and ruptured vesicles ultimately form scabs. Studies related to genotyping of GTPV isolates prevailing in the field condition are scanty in literature. Further, there is scanty literature pertaining to cross-border surveillance of the pathogen across the countries especially, between two neighbouring countries India and China covering an approximately 3,488 km of international border (Annual Report, 2007–2008). Hence, the study was planned in an anticipation of the applicability of the tetra-primer amplification refractory mutation system-polymerase chain reaction (TARMS-PCR) (Ye *et al.* 2001) in case of viruses for genotyping of single nucleotide polymorphism (SNPs). Though the technique is most often used for allele specific variations and SNP profiling in heterozygous system (Ahlawat *et al.* 2014, Guan *et al.* 2014; Singh *et al.* 2014; Wang *et al.* 2014; Zhang *et al.* 2015, 2013), but the same is rarely used among viral/bacterial genome. The same technique was further anticipated to screen a SNP in GTPV *p32* gene which could be used as a unique epidemiological marker. Our preliminary analysis showed that there is SNP variation in *p*32 gene among the GTPV data available in the NCBI GenBank. To study further, a TARMS PCR assay was developed and this technique was found unique in grouping allele specific GTPV isolates which has immense potential in cross-border surveillance among India and China.

Present address: [1]PhD Scholar (drmishraadarsh@gmail.com), Department of Veterinary Microbiology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai. [2]Director (parimalroy580@gmail.com), ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-INVEDI), Bengaluru, Karnataka.

MATERIALS AND METHODS

Virus isolates: A total of 11 GTPV field isolates/ positive clinical samples, i.e. GTPV3/WB/10, GTPV4/WB/10, GTPV11/WB/10, GTPV12/WB/10, GTPV13/WB/10, GTPV23/TN/15, GTPV24/TN/15, GTPV26/TN/15, GTPV27/TN/15, GTPV29/TN/15 and GTPV30/TN/15 were used for genotyping by a novel TARMS-PCR assay.

The isolates/clinical scab materials were confirmed as GTPV through the PCR-restriction fragment length polymorphism (RFLP) method of *p32* gene (Roy *et al*. 2017). All the isolates were handled with proper bio-safety precautionary measures.

Preparation of DNA template: The template DNA was prepared from field isolates and/or positive clinical scab samples collected from suspected disease outbreaks by QIAamp DNA mini kit (Qiagen, USA) following manufacturer's instructions.

Retrieval and analysis of p32 *gene sequences of goatpox virus:* The complete gene sequences of GTPV *p*32 gene available on NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) were retrieved for determining the consensus sequence and presence of single nucleotide polymorphisms (SNPs), if any. A total of 27 available *p*32 gene sequences including 23 complete gene sequences were retrieved. The accession no. of the sequences were HM572329.1/China, AY881707.1/China, AY159333.1/India, JN602370.1/China, JN596275.1/China, HM572331.1/China, EF522181.1/China, EF522180.1/China, EF522179.1/China, EF522178.1/China, EF522177.1/China, EF522176.1/China, KJ026560.1/China, KJ026559.1/China, KJ026558.1/China, KJ026557.1/China, KJ026556.1/China, KF468762.1/India, KF468759.1/India, KF468758.1/India, KF468757.1/India, FJ748488.1/India, AY382869.1/India, EF514892.1/China, EF514891.1/China, EF514890.1/China and EF514889.1/China.

Retrieved *p*32 gene sequences of Indian origin (7 numbers) and of Chinese origin (20 numbers) were analysed for multiple sequence alignment through ClustalW Multiple alignment programme of BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall, 1999). Presence of consensus sequences and mutations were analysed.

Designing of Primers: The primers were designed using software PRIMER1 (http:// primer1.soton.ac.uk) following earlier report of TARMS-PCR (Ye *et al.* 2001) from GTPV envelope protein (P32) gene sequence (NCBI GenBank accession number EF522177.1) and designated as forward inner primer (FIP), reverse inner primer (RIP), forward outer primer (FOP) and reverse outer primer (ROP) (Table 1) as per the strategy shown (Fig. 1). FIP corresponds to the 'T' allele and RIP corresponds to 'C' allele at 645th position of envelope protein (P32) gene.

Standardization: The TARMS-PCR reaction mixture was prepared comprising of 12.5 µl of master mix (Ampliqon), 0.5 µl each inner primers (10pm/µl), 1.0 µl each outer primers (10 pm/µl), 2.0 µl of template DNA. Final volume

Table 1. Sequence of primers used for tetra-primer ARMS-PCR

Primer Name	Sequence (5′–3′)	Oligo (bp)
FIP	TCTACCAGTTTGAGTTTTGAAATGGAT	27
RIP	TGAGTTTTAATTCTTTTTCCAACGTG	26
FOP	GAGGTAAAAAGTTCTATTGCAAAACACTT	29
ROP	ACGTAAATAACATACCTGCTAAAAACCA	28

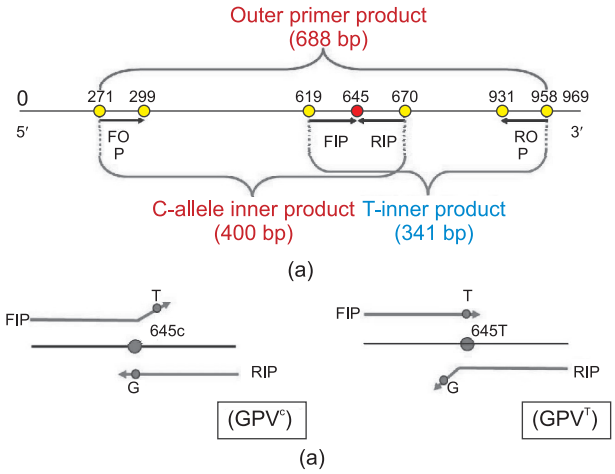

Fig.1. (a) Locations of primers FOP, ROP, FIP and RIP and corresponding amplification product sizes of tetra-primer ARMS-PCR; (b) Differential strategy of inner primers binding at the 645th nucleotide position of *p32* gene among the genotypes GPVC and GPVT.

was made to 25 µl by adding nuclease free water. The amplification condition comprised of 95°C for 5 min followed by 40 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. Amplification was carried out in thermal cycler (Eppendorf Pro-S, Germany). The amplified PCR products were run in ethidium bromide (0.5 µg/ml) stained 1.5% agarose gel, visualized and recorded by gel documentation system (Universal Hood II, BioRad, USA).

Sequencing: GTPV *p*32 gene was further sequenced and analyzed from randomly selected four isolates/samples for the confirmation of the accuracy of the technique. The *p32* gene of 966 bp was amplified following the protocol described (Bhanot *et al.* 2009) using 5′-ATGGCAGATATCCCATTATATG-3′ as forward primer and 5′-CAATAAATGCATATATCAA-3′ as reverse primer. The amplification condition comprised of 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 51°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were purified by mini elute gel extraction kit (Qiagen, Germany) following manufacturer's instructions and sequenced in 96-capillary ABI 3730xl DNA analyzer (Applied Biosystems) commercially through Sanger sequencing technique. Raw DNA sequencing data from a BigDye Terminator reaction was analyzed by ABI PRISM® DNA Sequencing Analysis Software v.5.0 Guide. The sequencing results were further analyzed through BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall, 1999).

RESULTS AND DISCUSSION

The GTPV produces disease with a mild to severe clinical implications primarily in goats and occasionally in sheep where both sheep and goats are flocked together. Sometimes it causes a severe mortality in affected flocks. So, the nature of the field outbreak should be thoroughly investigated for an efficient control strategy. More over the allele specific

distribution of the virus is also very little known which may be important for epidemiological surveillance. The *p*32 gene encodes envelope protein essential for attachment of the pathogen to host. The protein is found in membrane surface of mature intracellular virus particle (Tulman *et al.* 2002). It is one of the highly conserved proteins among the members (Hosamani *et al.* 2004). Any alteration in its coding sequence may result in the altered magnitude and severity of the infection. Similar kind of inference may be drawn in any other viral/bacterial diseases, where presence of a stable single-base-substitution may be responsible for altered protein function and nature of the pathogen as a whole. Moreover, any synonymous SNP, which can be used as an epidemiology surveillance marker are essential for disease monitoring and surveillance strategies.

The TARMS-PCR uses two pair of primers (inner and outer) for any single base substitutions resulting in any synonymous or non-synonymous SNPs. It results in amplification of one allele specific product of either of the alleles by either of inner primers and another common amplification product of outer primers. In this technique, a mismatch at 3′ end base of an inner primer (0^{th} position from 3′ end) to that of nucleic acid template confers allele specificity. In addition to this, another deliberate mismatch is included at -2 positioned nucleotide base from 3′ end in the inner primers, which increases the allele specificity (Ye *et al.* 2001). Usually, longer primers like that of 26 oligonucleotides or more are used to minimize the stability difference of primers annealed to the target or non-target alleles, facilitating the fact that allele specificity results from differences in extension rate, rather than hybridisation rate (Ye *et al.* 2001). In contrast to Bi-PASA (bidirectional PCR amplification of specific alleles) (Liu *et al.* 1997) and Tetra-primer PCR (Ye *et al.* 1992), the TARMS-PCR is more specific by taking into account a mismatch at -2 positioned nucleotide base from 3′ end. It is developed by Ye *et al.* (2001) by implementing certain principles of Tetra-primer PCR (Ye *et al.* 1992) with ARMS (Newton *et al.* 1989).

In the present study, 27 sequences of *p*32 gene of GTPV retrieved and analyzed, out of which, 12 sequences were found as T allele genotypes of goatpox virus ($GTPV^T$) and 15 sequences as C allele genotypes of goatpox virus ($GTPV^C$). Interestingly, all seven sequences of Indian origin were found to be of $GTPV^T$ type; whereas, among the twenty sequences of Chinese origin, fifteen sequences belonged to $GTPV^C$ category and only five sequences belonged to $GTPV^T$ type. It was inferred that Indian GTPVs are mostly of $GTPV^T$ type, whereas, Chinese GTPVs are of either category (Fig. 2).

Further, in the present report, TARMS-PCR was designed and standardized so as to get two amplified products of size 688 bp and 341 bp for $GTPV^T$ and 688 bp and 400 bp for $GTPV^C$ (Fig. 1). Here, the inner to outer primer ratio used during standardization were 10:1, 1:1, 1:2, 1:5, 1:10 and the most significant result was obtained in 1:2 ratio.

The developed technique was further assessed for its applicability using clinical field isolates. All the GTPV field

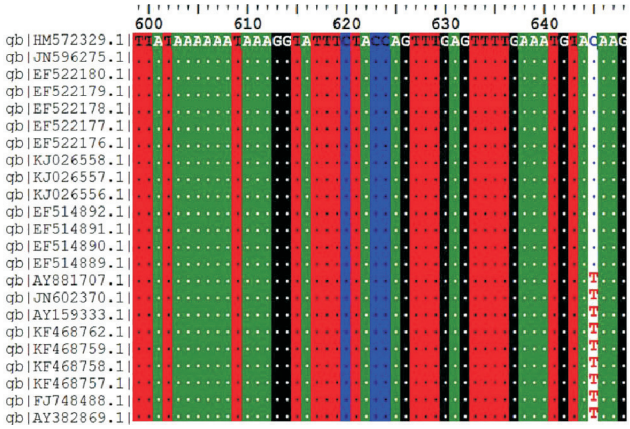

Fig. 2. Conservation plot obtained by ClustalW Multiple Sequence Alignment analyzed through BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall, 1999).

isolates were amplified and PCR products specific for $GTPV^T$ were obtained of 688 bp and 341 bp size. It indicated that all the Indian isolates taken in the study were having thymine at 645^{th} position of envelope protein (P32) gene. Further, the 966 bp product of *p*32 gene was sequenced. The 645^{th} nucleotide with single nucleotide polymorphism location was amplified within the region. Sequence data were submitted to NCBI Genbank with accession no. KU686998, KY614168, KY614170 and KY508697. Sequencing data had revealed the presence of thymine nucleotide at 645^{th} position in concordance to TARMS-PCR result.

The developed TARMS-PCR technique was proved to be an efficient tool for genotyping of GTPV field isolates. The Indian GTPV field isolates examined were found to be of $GTPV^T$ type whereas the Chinese origins were either $GTPV^T/GTPV^C$ type. Though this report preliminarily aimed at a synonymous SNP to understand the applicability of the technique, the same can be exploited for screening of any non-synonymous or expression SNPs. It was found to be a useful genotyping tool based on single nucleotide polymorphism to determine prevalence of an allele specific genotype in field condition. Such, genotyping will help in constant surveillance over the character of epizootic isolates or the emergence of other allele-variant ones. As two GTPV genotypes ($_{645}GTPV^{C/T}$) are found in China, this technique can be applied in future, for monitoring cross-border incursion of $GTPV^C$ genotype to India. This technique can be further applied to other such viruses to unveil the presence of any deleterious mutants and allelic variants in field conditions.

ACKNOWLEDGEMENTS

Authors highly acknowledge the Director, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University for providing necessary facilities for the research work.

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