B5R GENE BASED SEQUENCE ANALYSIS OF INDIAN BUFFALOPOX VIRUS ISOLATES IN RELATION TO OTHER ORTHOPOXVIRUSES

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Summary. – We determined complete nucleotide sequence of B5R gene homologue of Vaccinia virus (VACV) in five Buffalopox virus (BPXV) isolates of Indian origin. The obtained sequences were compared with themselves and with corresponding sequences of the other orthopoxviruses. Sequence analysis revealed 99.7–99.8% and 99.4–99.7% identities among the BPXV isolates for B5R gene at the nucleotide and amino acid levels, respectively. Sequence identities of B5R gene between BPXV and VACV isolates (98.1–99.7%) or other orthopoxviruses (95.6–99.2%) showed highly conserved nature of this protein and a closer relationship of BPXV isolates to VACV than to other orthopoxviruses.

Key words: Buffalopox virus; B5R gene; orthopoxviruses; sequence analysis

Introduction

Buffalopox is a zoonotic and contagious viral disease affecting buffaloes and rarely cows with high morbidity rate up to 80% and severe production losses (Lal *et al*., 1977; FAO/WHO technical report, 1967). BPXV (the species *Buffalopox virus*, the genus O*rthopoxvirus*) differs from VACV or Cowpox virus (CPXV) (van Regenmortel *et al*., 2000; Murphy *et al*., 1999). The disease is mainly prevalent in India and south Asia, but over the recent years, emergence of human and animal poxvirus infections caused by VACV like viruses Contagalo and Aracatuba have been reported in Brazil (Damaso *et al*., 2000; de Souza Trindade *et al*., 2003).

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Abbreviations: BPXV = Buffalopox virus; CMLV = Camelpox virus; CPXV = Cowpox virus; EEV = extracellular enveloped virus; ECTV = Ectromelia virus; IMV = intracellular mature virus; MPXV = Monkeypox virus; RPXV = Rabbitpox virus; SCR = short consensus repeat; VACV = Vaccinia virus; VARV = Variola virus

The protein encoded by B5R gene of poxviruses is a glycosylated membrane protein found on both the membranes of infected cells and extracellular enveloped virus (EEV), but not on intracellular mature virus (IMV). B5R gene of BPXV is 954 nt in length, encoding a putative polypeptide of 317 aa like in other orthopoxviruses (Herrera *et al*., 1998).

B5R is essential for efficient EEV formation and for trans-Golgi/endosomal membrane wrapping of IMV (Ward and Moss, 2001). Formation of EEV is essential for VACV spread *in vitro* and *in vivo* (Payne, 1980). Deletion of the B5R gene results in decreased EEV formation and B5R deletion mutants are highly attenuated *in vivo* (Engelstad and Smith, 1993; Parkinson and Smith, 1994). Antibodies to a specific protective epitopes on protein B5R were able to prevent infection with EEV indicating that B5R is likely to play a crucial role in the initial steps of VACV infection (Isaacs *et al*., 1992; Galmiche *et al*., 1999). Moreover, it may be involved in viral evasion from host immune responses (Isaacs *et al*., 1992; Martinez-Pomares *et al*., 1993).

B5R protein of VACV contains four copies of 50–70 aa repeats called short consensus repeat (SCR) in extracellular domain (SCR-1 to SCR-4, aa 20–236) that are similar to

SCRs present in complement regulatory proteins (Krych *et al*., 1992; Martinez-Pomares *et al.*, 1993). The function of B5R protein depends also on the other distinct domains of the protein e.g. signal peptide (aa 1–19), transmembrane domain (aa 276–303), and cytoplasmic tail. The domain SCR-2 contains three N-linked glycosylation sites (Herrera *et al*., 1998). To explore the putative variation, if any, between BPXV and VACV in the sequence of B5R protein, the present study reports sequencing of B5R gene of five BPXV isolates and comparative sequence analysis of these isolates to VACV and other orthopoxviruses

Materials and Methods

Cells and viruses. Five BPXV isolates, namely BP4-reference strain (Singh and Singh, 1967), VIJ96 – Vijaywada 1996; AUR – Aurangabad 2003; PUNE – Pune 2003; BANG – Bangalore 2004, from various geographical locations of India were propagated in Vero cells. The cells were grown in Eagle's MEM supplemented with 10% of newborn calf serum.

PCR, cloning and sequencing. Infected cells in 25 cm^2 tissue culture flasks showing >90% CPE were harvested in 250 µl of PBS pH 7.2 and used for extraction of total DNA with the AuPreP™ DNA Extraction Kit (Life Technologies, India). Viral DNAs were initially confirmed by diagnostic PCR using specific primers for orthopoxviruses (CoPV3 and CoPV4) for amplification of a 552bp fragment of inclusion gene.

Further, the DNA samples were subjected to a PCR amplifying full-length B5R gene using the following primers designed according the sequence of VACV Copenhagen strain (Acc. No. M35027): B5R Full Forward (5'-GTTTCCTTTTAGTGCTCGA CAGTGT-3') and B5R Full Reverse (5'-AACGGATTTATATT CACGGTAGCA-3') (Funahashi *et al*., 1988; Meyer and Rziha, 1993). The PCR consisted of initial denaturation at 94°C for 3 mins, 35 cycles of 94°C/1 min, 60°C/1 min, and 72°/1 min, and final extension at 72°C for 10 mins. The PCR products of 1019 bp were gel purified using the AuPreP PCR^{Gelx} Kit and cloned into the pGEM-T easy vector (Promega). The recombinant plasmids were isolated, verified by PCR and restriction analysis, and sequenced using an automated DNA sequencer (ABI 3100; Perkin Elmer).

Sequence analysis. An ORF sequence homology search using NCBI BLAST server was performed and the sequence identity among BPXV isolates and other poxviruses was determined (Altschul *et al*., 1997). Deduced amino acids sequences were aligned using the Clustal W program of Lasergene 6.0 (DNASTAR Inc., USA). Phylogenetic analysis was carried out using MEGA version 3.1 (Kumar *et al.,* 2004), using the neighbor joining method, and the tree topologies were evaluated using 1000 replicates of the data set.

We compared sequences of B5R gene of five BPXV isolates with 12 representative orthopoxviruses (Vaccinia virus (VACV) strains-Ankara (ANK), U94848; Western Reserve (WR), AY243312; Tian Tan (TT), AF095689; Modified virus Ankara (MVA), AY603355; LC16mo, AY678277; Cowpox virus (CPXV) strains-Brighton Red (BR), AF482758; GRI-90, X94355; Camelpox virus (CMLV)-M-96 Kazakhstan, AF438165; Monkeypox virus (MPXV)-Zaire-96-I-16 (ZR), AF380138; Rabbitpox virus (RPXV), AY484669; Ectromelia virus (ECTV)-Moscow (Mos), AF012825; Variola virus (VARV)-India-1967, ssp. major (IND 67), X69198).

Results and Discussion

We determined the full-length sequences of B5R gene homologue of VACV-Copenhagen in BPXV isolates. The analyzed gene was amplified using DNA isolated from five BPXV isolates to yield a product size of 1019 bp (data not shown). Tested BPXV isolates showed 99.7–99.8% and 99.4–99.7% sequence identity among themselves at the nt and aa levels, respectively. High nt sequence identity of the BPXV-B5R gene between VACV (98.1–99.7%) and other analyzed orthopoxviruses (95.6–99.2%) showed highly conserved type of protein. At the aa level, BPXV-B5R protein showed a high degree of sequence homology with VACVs (97.2–100%) and other orthopoxviruses (92.5–98.4%). The detected homologies were for RPXV – 98.4%; MPXV – 96.2%; CPXV – 92.8–96.5%; ECTV – 94.7%; VARV – 92.8% and for CMLV – 92.5%.

Detected sequence identities showed, that B5R genes were relatively conserved within the genus *Orthopoxvirus* (Fig. 1). In the multiple aligned sequences of amino acids (Fig. 1), many substitutions were unique only to BPXV and VACV isolates. These substitutions included D40N, K41N and L55S in SCR-1, V82I and D87N in SCR-2 domains and E243K in the transmembrane domain. SCR-1 domain (aa 20–71) of the BPXV isolates, which was composed mostly of hydrophilic amino acids and shared a 100% homology with VACV-ANK and VACV-MVA strains.As expected, SCR-2 domain (aa 75–124) of BPXV isolates also contained three potential glycosylation site motifs (positions aa 94–96, 116–118, 120–122), like in all analyzed orthopoxviruses.

SCR-3 domain (aa 129–181) of BPXV-B5R isolates had complete sequence homology with VACV-B5R strains. It shared nearly 100% homology (except aa change M153I) with VACV strains, while only 92% identity with other orthopoxviruses. Mutagenesis studies revealed that amino acids residues required for Golgi membrane localization and efficient plasma membrane retrieval of the VACV-B5R protein consisted of Y at position 310 and two adjacent L residues at positions 315 and 316 in the transmembrane domain (Ward and Moss, 2000). The same amino acids at the same positions were also found in BPXV-B5R proteins as well as in B5R proteins of other orthopoxviruses.

The phylogenetic analysis of B5R protein of BPXV isolates with other orthopoxviruses at the aa level reaffirmed

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>            { Signal peptide  }{Extracellular domain ->
>                                [                        (SCR-1)
>           1       10        20        30        40        50        60        70
BPXV BP4     MKTISVVTLLCVLPAVVYSTCTVPTMNNAKLTSTETSFNNNQKVTFTCDQGYHSSDPNAVCETDKWKYEN
BPXV VIJ 96  ............................................................................
BPXV AUR     ............................................................................
BPXV PUNE    ............................................................................
BPXV BANG    ..............................................I.............................
VACV MVA     ............................................................................
VACV Ankara  ............................................................................
VACV WR      ...................................DK.........................................
VACV TT      ...................................DK.........................................
VACV LC16mO  ...................................DK............L..............................
CPXV BR      ...................................DK........S..Y.L...........T...
CPXV GRI-90  ...................................DK............L..............................
CMLV M-96    ...................................DK........S..Y.L..............................
MPXV ZR      ...................................DK........S....L..............................
RPXV         ............................................................................
EV Mos       ...................................DKH...........L..............................
VARV         ...................................DK........S..Y.L..............................

>                              <- Extracellular domain ->
>            ]    [                 (SCR-2)                        ]    [
>           71       80        90       100       110       120       130       140
BPXV BP4     PCKKMCTVSDYISELYNKPLYEVNSTMTLSCNGETKYFRCEEKNGNTSWNDTVTCPNAECQPLQLEHGSC
BPXV VIJ 96  ............................................................................
BPXV AUR     ............................................................................
BPXV PUNE    ............................................................................
BPXV BANG    ............................................................................
VACV MVA     ............................................................................
VACV Ankara  ............................................................................
VACV WR      ............................................................................
VACV TT      ............................................................................
VACV LC16mO  ...........V....D.....................................................
CPXV BR      ...........V....D.........I..T.KD.............................R......
CPXV GRI-90  ...........V....D.....................................................
CMLV M-96    ...........V....D.......A.I..I.KD...........E.................S........
MPXV ZR      ...........V....D.....................................................
RPXV         ............................................................................
EV Mos       ...........V....D...........R..S.............................R......
VARV         ...........V............AII..I.KD.............................S...D....

>                              <- Extracellular domain ->
>                        (SCR-3)                      ][             (SCR-4)
>          141      150       160       170       180       190       200       210
BPXV BP4     QPVKEKYSFGEYITINCDVGYEVIGASYISCTANSWNVIPSCQQKCDIPSLSNGLISGSTFSIGGVIHLS
BPXV VIJ 96  ............................................................................
BPXV AUR     ...................................S........................................
BPXV PUNE    ............................................................................
BPXV BANG    ............................................................................
VACV MVA     ............................................................................
VACV Ankara  ............................................................................
VACV WR      ...........M..................................M.....................
VACV TT      ...........M..................................M.....................
VACV LC16mO  ...........M..................................M.....................
CPXV BR      ...........H......L.....................................................
CPXV GRI-90  ...........M.....................................................
CMLV M-96    ...........H.......................I.....................................
MPXV ZR      ...........M...........V.....................................
RPXV         ...........M.....................................................
EV Mos       ...........M.....................................................
VARV         ...........H................T.....................................
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Fig. 1

Comparison of B5R protein amino acid sequences of five BPXV isolates with other orthopoxviruses

Identical (dot) and substituted (code) amino acid in relation to BPXV-BP4. The domains of the B5R protein of VACV-WR are marked as lines above the sequence of BPXV BP4.

that BPXVs clustering with VACVs, particularly VACV-MVA and VACV-ANK strains were closely related to VACV as reported previously (Singh *et al*., 2006). BPXVs were clustered with VACVs with 98% bootstrap confidence, but they were different from other orthopoxviruses (data not shown). Data supports earlier observation that the virus is a clade of VACV (Murphy *et al.,* 1999).

Earlier studies based on genomic restriction enzyme polymorphism studies did not show any significant differences between different VACV and BPXV isolates (Dumbell and Richardson, 1993). Also, comparative analysis between VACV and BPXV envelope protein genes revealed a very close relationship (Singh *et al*., 2006).

Based on the sequence analysis of the B5R gene, we found very close relationship of BPXV isolates to VACV strains. Close similarity of BPXV and VACV isolates at nt and aa levels implied that BPXV-B5R protein could play a similar role as that of VACV in the pathogenesis. Monitoring of the minor changes at nt level in BPXV isolates, can predict corresponding protein structural changes and also the changes in biological activities of circulating BPXVs.

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