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Immunogenicity and protective efficacy of recombinant major envelope protein (rH3L) of buffalopox virus in animal models

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ABSTRACT

Buffalopox virus, a zoonotic Indian vaccinia-like virus, is responsible for contagious disease affecting mainly buffaloes, cattle and humans. H3L gene, encoding for an immunodominant major envelope protein of intracellular mature virion of orthopoxviruses, is highly conserved and found to elicit neutralizing antibodies. Therefore in the present study, the immunogenicity and protective efficacy of the recombinant H3L protein of buffalopox virus in laboratory animal models has been evaluated. A partial H3L gene encoding for the C-terminal truncated ectodomain of H3L protein ($_{1}$M to I_{280}) of BPXV-Vij/96 strain was cloned, over-expressed and purified as histidine-tagged fusion protein (50 kDa) from *Escherichia coli* using Ni-NTA affinity chromatography. The purified rH3L protein was further used for active immunization of guinea pig (250 μg/dose) and adult mice (10 μg and 50 μg/dose) with or without adjuvants (alum, Freund's Complete Adjuvant and CpG). Subsequently, a gradual increase in antigen specific serum IgG as well as neutralizing antibody titres measured by using indirect-ELISA and serum neutralization test respectively, was noted in both guinea pigs and mouse models. Suckling mice immunized passively with anti-H3L serum showed 80% pre-exposure prophylaxis upon challenge with virulent buffalopox virus strain. An indirect-ELISA based on rH3L protein showed no cross-reactivity with hyperimmune sera against sheeppox virus (SPPV), goatpox virus (GTPV), orf virus (ORFV), foot- and-mouth disease virus (FMDV), *peste des petits ruminants* virus (PPRV) and bluetongue virus (BTV) during the course of study. The study highlights the potential utility of rH3L protein as a safer prophylactic and diagnostic reagent for buffalopox.

1. Introduction

Buffalopox, an emerging/re-emerging contagious viral zoonotic infection, is caused by buffalopox virus (BPXV) which is an Indian close variant of vaccinia virus (VACV), belongs to the genus *Orthopoxvirus* (OPV) of family *Poxviridae* (Bhanuprakash et al., 2010; King et al., 2012). Globally, emergence and re-emergence of the Vaccinia-like viruses (VLVs) such as Aracatuba, Cantagalo, Guarani and Passatempo viruses in Brazil, has been a major concern in recent times (Essbauer et al., 2010; Singh et al., 2012) and especially, BPXV in Indian subcontinent including India, Pakistan, Nepal and Bangladesh. Among VLVs, BPXV is known to affect primarily buffaloes, cattle and occasionally in-contact humans (WHO, 1967; Singh et al., 2012). The disease in animals is associated with high morbidity and significant productivity loss. VACV, a close clade of BPXV consists of linear double stranded DNA (~190 kbp) which encodes for >200 proteins (Murphy et al., 1999). BPXV has shown ultra-structural similarities with VACV which produces both extracellular enveloped virions (EEV) and intracellular mature virions (IMV) each with unique proteins (Chertov et al., 1991; Chung et al., 2006). IMV is most abundant form with a capacity to generate potent neutralizing antibodies. Moreover, molecular characterization based on selected genes of IMV namely A27L, H3L, and D8L encoding for major envelope proteins revealed higher sequence identity (>99%) of BPXV isolates with that of VACV (Singh et al., 2006a). H3L is among few proteins of VACV known to induce

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neutralizing antibodies (Ramirez et al., 2002). H3L encoded p35 (324 aa; 35 kDa) is an immunodominant integral major envelope protein found on IMV of VACV (Zinoviev et al., 1994) and mediates VACV adsorption to cell surface heparan sulfate and is also involved in virion morphogenesis (Lin et al., 2000).

Currently, no commercial effective vaccine is available for immunization of buffaloes in endemic areas to protect against buffalopox despite several attempts in the past (Mohanty and Rai, 1989, 1992). However, various candidate proteins of VACV including H3L have been studied in animal models. Notably, immunogenicity and protective efficacy of recombinant H3L protein of VACV (Davies et al., 2005) and passive protection efficacy using immune serum (Davies et al., 2005) or monoclonal antibodies (McCausland et al., 2010; Crickard et al., 2012) to recombinant H3L have been studied and found to provide protection in animal models. Despite the similarity of BPXV-H3L protein with that of VACV, no attempts were made to ascertain its immunogenicity and protective efficacy following virulent BPXV challenge. An efficient and protective buffalopox vaccine which is novel and potent is most warranted for use in endemic areas.

Generally, laboratory diagnosis of buffalopox is based on virus isolation, electron microscopy (EM), serum neutralization test (SNT), counter-immunoelectrophoresis (CIE) and molecular techniques including conventional polymerase chain reaction (PCR) and TaqMan probe-based quantitative real-time PCR (Singh et al., 2007; Bhanuprakash et al., 2010). Although, whole virus antigen based indirect-ELISA assays were developed in the past (Mohanty and Rai, 1990; Ghildyal et al., 1986), there is a greater zoonotic risk of handling live culture, which paved the way for alternate strategies under recombinant DNA technology.

In view of productivity losses caused by buffalopox infection in animals and its increased zoonotic impact on public health in recent times, there is an imminent quest to design and develop improved diagnostics and prophylactic/control strategies. A non-infectious alternative subunit vaccine formulation and a potential diagnostic antigen based on recombinant protein will be of potential utility in the control of buffalopox. In this study, we report, for the first time, immunogenicity (both active and passive), protective efficacy and diagnostic specificity of recombinant H3L protein of BPXV as a candidate antigen for prophylaxis as well as diagnosis.

2. Materials and methods

2.1. Virus

BPXV-Vij/96 P50 (vaccine strain) and BPXV-Aurangabad/04, P4 (virulent strain), and Vero cells (ATCC, CCL81) maintained at Pox Virus Laboratory, Division of Virology, ICAR-IVRI, Mukteswar, India, were used for propagation of BPXV.

2.2. Protein (BPXV-H3L) sequence analysis and construction of expression clone

H3L gene of BPXV-Vij/96 was used for prediction of protein characteristics using proteomic tools such as PROTEAN program (DNASTAR), PSIPRED, PredictProtein and ProtParam tools from ExPaSy website (Jones, 1999; Rost et al., 1996). A set of primers targeting H3L gene of BPXV-Vij/96 P50 (vaccine strain) encoding for C-terminal ($_{281}$S–I$_{324}$) truncated H3L protein (1-840 nt and $_{1}$M-I$_{280}$ aa region) were designed based on its available sequence (Accession # DQ117955) in GenBank (Singh et al., 2006a). The forward primer, H3L-F: 5'-gcta*CCATGG*CGGCGGTGAAAACTCCTGTTAT-3'; and reverse primer, H3L-TM-R: 5'-gtg*CTCGA*GAATCAGTGGAGTAGTAAACGCGTA-3′, had added restriction enzyme sites (italics) for *Nco*I and *Xho*I, respectively at 5′ end along with primer tags (small letters). The required primers were synthesized (Metabion International, Germany) and procured.

The BPXV-Vij/96 strain was propagated in Vero cells maintained at the repository and used for DNA extraction using AuPrep GEN-DNA Extraction kit (Life Technologies, India). The PCR reaction mixture (25 μl) comprised of template DNA of BPXV, 10 pmol of each primer, 10 mM of each dNTPs, 10x PCR buffer, 25 mM $MgCl_2$ and 1 U of *Taq*DNA polymerase; and amplification conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The purified PCR product and pET32a vector (Novagen, USA) were digested with *Nco*I and *Xho*I; and ligated recombinant plasmids were initially transformed into *E. coli* Top 10F' strain (Invitrogen, USA). Further, positive clone was transferred into expression host *E. coli* Origami (DE3) pLacI cells (Novagen, USA) and selected using antibiotics such as ampicillin (50 μg/ml) and chloramphenicol (35 μg/ml).

2.3. Expression, purification and western blot analysis

E. coli Origami (DE3)pLacI cells harboring recombinant plasmid were grown on 1 L Luria Bertani (LB) broth containing appropriate antibiotics at 30 °C to an O.D. of 0.4–0.6 before induction with 1 mM IPTG and harvested at 3 h post induction. The rH3L protein was purified under both native/denaturing conditions by affinity chromatography using Ni-NTA superflow cartridges (Qiagen, USA) as described earlier (Kumar et al., 2013; Shivachandra et al., 2012, 2014, 2015; Kumar et al., 2015). Following column binding, washing and elution, peak protein fractions were pooled, dialyzed against a buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl). Further, concentrated (Vivaspin, Germany) protein aliquots were quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) before storage at −80 °C until further use.

For confirmation of rH3L protein, induced/un-induced *E.coli* cultures as well as purified protein fractions were separated by 10% SDS-PAGE and subsequently transferred on to nitrocellulose membrane using semi dry immunoblot system (Amersham Pharmacia, USA). Detection was carried out using 1:100 diluted anti-BPXV/anti-CMLV polyclonal hyperimmune rabbit serum (available at authors' laboratory as they were produced previously in rabbits using respective purified viruses) as primary antibody and 1:16,000 diluted goat anti-rabbit IgG horseradish peroxidase (HRPO) conjugate (Sigma, USA) as secondary antibody before developing a blot using DAB substrate (Sigma, USA) and analysis of results.

2.4. Animal immunization and immune response evaluation

Healthy Duncan Hartley guinea pigs (7–8 months old), Swiss albino adult mice (6–8 week old) and suckling mice (5–6 days old) reared in pathogen-free environment at Laboratory Animal Section, ICAR-IVRI, Mukteswar, India, were used in immunization trials. All laboratory animal experiments were conducted according to the norms of 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)', Ministry of Environment and Forests, Animal Welfare Division, Government of India and approval by the Institutional Animal Ethics Committee (IAEC) as well as Institutional Biosafety Committee (IBSC), ICAR-IVRI, Mukteswar, Uttarakhand, India. The animals were maintained under standard husbandry conditions like humidity, temperature and photo period of 12 h. Pellet diet and water were given *ad libitum*.

2.4.1. Active immunization of mice and guinea pigs

The animal experiment details are mentioned in Table 1. Briefly,