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Cloning of the transmembrane glycoproteins G and F from a Brazilian isolate of bovine respiratory syncytial virus in a prokaryotic system

[*Clonagem das glicoproteínas transmembrana G e F de um isolado brasileiro do vírus respiratório sincicial bovino em sistema procarioto*]

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ABSTRACT

The aim of this work was the cloning of those transmembrane glycoproteins G and F from an isolate bovine respiratory syncytial viruses (BRSV) – a Brazilian isolate of BRSV, named BRSV-25-BR in previous studies, in a prokaryotic system to proceed the sequencing of larger genomic fragments. The nucleotide substitutions were confirmed and these clones may also be used in further studies regarding the biological effects of those proteins *in vitro* and *in vivo*.

Keywords: bovine respiratory syncytial virus, calves, glycoproteins G and F

RESUMO

O objetivo deste trabalho foi a clonagem das glicoproteínas transmembrana G e F de um isolado de vírus respiratório sincicial bovino (BRSV) - um isolado brasileiro denominado BRSV-25-BR- que já demonstrou possuir mutações em regiões altamente conservadas do gene da proteína G - em sistema procariótico, com o intuito de sequenciar fragmentos genômicos maiores. As substituições de nucleotídeos foram confirmadas e tais clones podem ser utilizados em futuros estudos sobre os efeitos biológicos destas proteínas tanto in vitro *como* in vivo.

Palavras-chave: vírus respiratório sincicial bovino, bezerros, glicoproteínas G e F

INTRODUCTION

Bovine respiratory syncytial virus (BRSV) was first isolated in 1967 (Paccaud and Jacquier, 1970) from calves presenting respiratory distress. The distribution of BRSV is worldwide and it causes severe respiratory disease in young calves, characterized by bronchiolitis and interstitial pneumonia. BRSV is closely related to human respiratory syncytial virus (hRSV), which is a major cause of respiratory disease in young children, and the epidemiology and pathogenesis of infection for these viruses are similar (Valarcher and Taylor, 2007).

The BRSV belongs to the genus *Pneumovirus*, subfamily *Pneumovirinae*, family *Paramyxoviridae*, *Mononegavirales* order (Vainionpää *et al*., 1989; Larsen, 2000; Easton *et al*., 2004). Members of the *Mononegavirales* order possess genomes constituted by a single strand of negative-sense RNA (5' → 3' sense). The subfamily *Pneumovirinae* of *Paramyxoviridae* has two genera: the genus *Pneumovirus* contains bovine (RSV) as well as

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human, caprine, and ovine RSVs and pneumonia virus of mice; the genus *Metapneumovirus* consists of avian metapneumovirus (aMPV) and human metapneumovirus (hMPV). The genus *Metapneumovirus* differs from the genus *Pneumovirus* on the absence of non-structural proteins NS1 and NS2, and in gene order for the F and M2 genes (Collins *et al*., 2001).

The BRSV virion consists of a nucleocapsid contained within a lipid envelope. The envelope is a lipid bilayer that is derived from the host plasmatic membrane. It contains three virally encoded transmenbrane surface glycoproteins; the attachment protein (G), the fusion protein (F), and the small hydrophobic SH protein (Collins *et al*., 2001).

The G protein was identified as the major attachment protein because antibodies specific to the G protein blocked the binding of the virus to cells (Levine *et al*., 1987), playing an important role in the immune response against BRSV infection (Bastien *et al*., 1997; Buchholz *et al*., 2000; Spilki *et al*., 2006a). The G protein is a heavily glycosylated, type II membrane protein that does not share any sequence or structural homologies with attachment proteins of other paramyxoviruses (Wertz *et al*., 1985) and lacks haemaglutinating and neuraminidase activities (Easton *et al*., 2004).

Glycoprotein G is composed of three domains: cytoplasmatic (AA 1-37) transmembrane (AA 38-65) and extracellular ectodomain (AA 66-257) (Langedijk *et al*., 1996). The cytoplasmatic domain is placed inside the viral envelope; the transmembrane domain runs across the cytoplasmatic membrane envelope. Both these sections of the protein are considerably conserved (Stine *et al*., 1997; Valentova, 2003). The extracellular domain of glycoprotein G is responsible for binding the virus to the sensitive cells, and the greatest differences within all viral proteins are localized there (Lerch *et al*., 1990). The ectodomain of the virus consists of hydrophobic variable regions and a central conserved region (Doreleijers *et al*., 1996; Langedijk *et al*., 1996). The variable regions of the protein are called mucin-like, due to their high content of carbohydrate side-chains bounded to Ser, Thr (O-linked sugars), and Pro (N-linked sugars). The central conserved region is the only relatively fixed and folded part of the ectodomain of RSV-G, containing four conserved cysteine residues which can form two disulphide bridges. It is speculated that this structure may interact with the host immune system, due to its similarity with fractalkines (Doreleijers *et al*., 1996; Langedijk *et al*., 1996). A Brazilian isolate has a major substitution of amino acids in this same region (Spilki *et al*., 2006a), and studies using this peptide may be of interest to discover whether these features may induce differences in the biological effects of the protein *in vivo*.

Experiments with mutant viruses possessing an induced deletion glycoprotein G coding gene showed that this glycoprotein is not necessary for virus replication *in vitro*, although its replication is considerably decreased in both cell cultures and the respiratory tract of mice. It also seems that glycoprotein G is not the only virus protein with attachment activity, but both the glycoproteins G and F play a role in attachment of the virus to cells (Karger *et al*., 2001; Techaarpornkul *et al*., 2001; Teng and Collins, 2002).

The F protein directs viral penetration through the fusion between the virion envelope and the host cell plasma membrane, delivering the nucleocapsid to the cytoplasm. Later in infection, the F protein expressed on the cell surface can mediate fusion with neighboring cells and form syncytia. This is a prominent cytophatic effect and is probably an additional mechanism of viral spread (Collins *et al*., 2001). The protein is synthesized primarily as the uncleaved precursor F_0 that is cleaved by cellular proteases in the trans-Golgi network to yield the disulfide-linked heterodimer F_2-F_1 (Collins *et al*., 2001). The F protein induces neutralizing antibodies and confers resistance to BRSV infection (Taylor *et al*., 1992; Taylor *et al*., 1997; Thomas, *et al*., 1998; Taylor *et al*., 2005). The F protein in BRSV is 574 AA in length (Lerch *et al*., 1990; Pastey *et al*., 1993) and is highly conserved between different BRSV isolates.

The objectives of the present work were to clone the transmembrane glycoproteins G and F from a Brazilian BRSV isolate (antigenic subgroup B) in a prokaryotic system, to examine the nucleotide sequences on the conserved central region G and on particular sites of the F protein in BRSV, furthermore, to show mutations which

are located in the conserved central region G protein demonstrated in previous work and to examine whether there were mutations on the F protein gene.

MATERIAL AND MEHTODS

The Brazilian strain BRSV-25-BR (Arns *et al*., 2003), was propagated in CRIB cells (Flores and Donis, 1995), a clone of MDBK cells resistant to the infection with BVDV, which is highly susceptible to the infection with BRSV (Flores and Donis, 1995; Spilki *et al*., 2006b). Cells were cultivated in Eagle's minimal essential medium (E-MEM) supplemented with 10% of Fetal Bovine Serum, free of antibiotics following routine protocols, and titrated through the visualization of syncytia formation in an inverted microscope (Zeiss Axyovert™, Oberkochen, Germany).

RNA was extracted from infected CRIB cultures using High Pure Viral Nucleic extraction kit™ (Roche Diagnostics™, Manheim, Germany). cDNA was synthesized using SuperscriptIII FirstStrand Synthesis Super Mix™ (Invitrogen Ltd., California, USA) following kit instructions for use with random hexamers. Primer oligonucleotides were originally designed for the present work. PCR of BRSV genes were amplified using specific primers designed for the 3' and 5' flanking regions of the G protein gene, which was the target for polymerase chain reaction. The forward primer BRSVG5 (5' ATG TCC AAC CAT ACC CAC CAT CT 3'); the reverse primer BRSVG3 (5' GGT GAA GAG AGG ATG CCT TG 3') corresponding to amplified products of 752 bp.

For the F protein gene the forward primer BRSVF5 (5' ATA AGG ATG GCG ACA ACA GC 3'was used, annealing to the 3' F protein gene flanking region); the reverse primer BRSVF3 (5' AGG CCA GGC TTT GGT TTA TT 3'), near to the 5' F protein gene flanking region, corresponds to amplified products of 1538 bp.

PCR was done in a 5µL mixture containing 2.5µL of 10X PCR buffer (10mµ Tris–HCl Ph 8.0, 50 mµ KCl and 0.2mµ MgCl2), 1µL of a 10mµ dNTPs mixture, 10mµ of each primer, 2.5mL of cDNA, and 0,2µL (5U/µL) of *Platinum*™ *High Fidelity* DNA Polymerase (Invitrogen Ltd., California, USA). The amplification was pre-heated for 5min at 95ºC, following for 35 cycles, each of them composed of 1min at 94ºC (denaturation), 1min at 56ºC (annealing), and 1min at 72ºC (extension). After the completion of the 35 cycles, a final extension of 7min at 72ºC was performed. Amplifications were carried out in a thermal cycler PCR System 9700 (Gene Amp, Applied Biosystems, Perkin-Elmer, California, USA). The PCR products were run on 1% agarose gel and visualized under UV light after being stained with ethidium bromide.

The cloning of PCR product G and F genes was performed by using the plasmid vector pCR®8/GW/TOPO® TA Cloning® (Invitrogen Ltd., California, USA), procedures were performed according to the manufacturer's instructions.

The cloning reactions pTOPOG and pTOPOF, which transformed pCR®8/GW/TOPO® TA Cloning® construct into competent *Escherichia coli* DH5α (Clontech, Heidelberg, Germany), following the transform heat-shock. The bacterial colonies containing recombinant plasmids were selected in LB medium containing antibiotic *spectinomycin* (Sigma) and the plasmid DNA was extracted and purified using by Kit *PureLink™ Quick Plasmid Miniprep Kit (*Invitrogen Ltd., California, USA), according to the manufacturer's instructions. The presence inserts pTOPOG and pTOPOF, were confirmed by PCR, following the protocol described.

The confirmation of the identity and orientation inserts, the PCR products and pTOPOG and pTOPOF were sequenced using specific primers G and F genes and primers M13 vector were sequenced three times each, both in forward and reverse directions using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems™).

The BioEdit software, version 7.0.5.2 (Hall, 1999), was used to manipulate the nucleotide sequences. Nucleotide alignments were performed using the Clustal W software, version 1.83 (Thompson *et al*., 1994). Similarity to the PCR products and inserts with complete gene sequences are deposited in *GenBank (*http://www.ncbi.nlm.nih.gov/Genbank/index.html). The BRSV nucleotide sequences and

pTOPOG and pTOPOF nucleotide sequences obtained were submitted to the *GenBank* with access numbers FJ543090 and FJ543091.

RESULTS AND DISCUSSION

The obtained PCR products (Figure 1) were cloned into vector pTOPO generating pTOPOG and pTOPOF. The nucleotide sequences G and F genes were obtained through the partial sequencing of the plasmids pTOPOG and pTOPOF with primer M13. Obtained nucleotide sequences were compared with homolog sequences deposited at *GenBank* using the NCBI BLAST N program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The similarity sequences have demonstrated the higher degree homology 98% for G protein gene and 96% for F protein gene, thus confirming the identity of the target genomic inserts.

Some differences were observed in the deduced amino acid level on the immunodominant region of the G gene. The two cysteins in positions 173 and 176 of the amino acid sequence were replaced by alanine residues, and there were also other substitutions at this site: Ser^{174} was replaced by another alanine, Thr^{175} was substituted for a hystidine and Glu^{177}, and Gly^{178} was replaced by lysine and arginine residues, respectively (Spilki *et al*., 2006c). The amino acid changes were examined for putative structural changes on the central domain of the protein, and the result is that a major substitution occurred in the cystine-noose coding region of the Brazilian strain BRSV-25-BR of the G protein (Spilki *et al*., 2006c).

The mutations described earlier for the G gene protein based on a smaller fragment which was sequenced directly from PCR products were now confirmed using plasmid-cloning based sequencing. No relevant mutations were found on the F protein gene.

In this work the cloning and prokaryotic sistem was described for the transmembrane glycoproteins G and F of BRSV, wich are the main targets of immune response. The neutralizing antibodies are induced by G and F proteins, as evaluated in calves vaccinated with recombinant vaccinia viruses (rVV) encoding these proteins. The high titre of neutralizing antibodies are induced by F protein, but only a low level of complement-dependent neutralizing antibodies are induced by G protein (Taylor *et al*., 1997; Thomas *et al*., 1998). These antibodies provide predominantly strain-specific immunity, although recombinant vaccines which express only G protein BRSV are able to induce complete protective immunity of the lower and to a lesser extent upper respiratory tract (Shirijver *et al*., 1996; Taylor *et al*., 1998).

The ability to manipulate the genome of BRSV may increase the understanding of the role of different proteins in the pathogenesis of this virus in calves and has provided opportunities for the development of stable, live attenuated virus vaccines, vector vaccines and DNA vaccines. Especially regarding the G protein of the isolate presently cloned, this may be useful to check whether particular features of this protein in such viral isolate may play a biological role or not, since previous work has shown that there is a major mutation between amino acid residues 173 and 178, within the central hydrophobic conserved region, exactly on the site of two of the four cysteine-noose forming cysteine residues, which may interact with the cellular receptor (Valarcher *et al*., 2000) and plays an important role in the induction of antiviral host immunity (Langedijk *et al*., 1997).

It may be speculated that the mutations presented by BRSV-25-BR G protein may have been induced by the presence of active and passive naturally acquired immune response against viral infection itself, and the virus may have been selected by infection animals processing antibodies to the virus, which selects antibody escape mutants, as demonstrated *in vitro* for hRSV (Martinez *et al*., 1997; Melero *et al*., 1997; Spilki *et al*., 2006c).

Despite these alterations on the central hydrophobic region of the attachment protein, this virus infects calves efficiently under experimental conditions inducing moderate lesions on the lungs of infected animals (Almeida *et al*., 2005; Spilki *et al*., 2006c).

An optimal BRSV vaccine would combine genetic stability, satisfactory growth *in vitro*, high attenuation *in vivo* and a satisfactory level of immunogenicity. Future studies with recombinant viruses represent a particularly

promising avenue for the development of vaccines and research, expressing antigens by virus vectors has proven to be a productive approach for studying in *vitro* and in *vivo* immune responses. An important feature of almost all recombinant viruses is the ability to induce not only humoral, but also cell-mediated immunity (Imler, 1995). Another possible use for the genes cloned on the present work may be as tools for the construction of recombinant vaccine candidates aiming BRSV immunization.

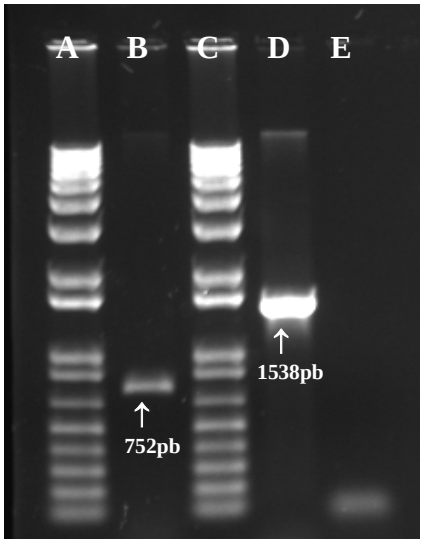

Figure 1. Bovine respiratory syncytial virus G and F gene amplification. RT-PCR was performed in RNA extracted from strain BRSV-25-BR infected CRIB cells for G and F genes. 1% agarose gel and visualized under UV light after being stained with ethidium bromide. A. 1Kb DNA Ladder *(*Invitrogen Ltd., California, USA); B. strain BRSV-25BR G gene; C. 1Kb DNA Ladder *(*Invitrogen Ltd., California, USA); B – strain BRSV-25BR F gene and E. Reaction control.

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