Identification of deletion mutant Respiratory Syncytial virus strains lacking most of the G-protein in immunocompromised children with pneumonia in South Africa

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Abstract
Respiratory syncytial virus G-protein deletion mutants replicate effectively in vitro but have not been detected in nature. Subtyping of RSV strains in hospitalized children in South Africa identified G-protein PCR amplicons significantly reduced in size in 2 out of 209 clinical specimens screened over 4 years. Sequence analysis revealed subtype B strains lacking
nearly the entire G protein ecto-domain in one HIV positive and one HIV exposed child hospitalized with pneumonia. The association of clinical strains lacking most of the G-protein with lower respiratory tract infection in immunocompromised children may have implications for RSV vaccine development.

Respiratory Syncytial Virus (RSV), a Pneumovirus, family Paramyxoviridae, is a major cause of pneumonia in children. Two antigenic subgroups (A and B) exist with partial cross protection(2). Neutralizing antibodies are directed against the F and G surface glycoproteins. Major antigenic differences are features of the G protein, a type II transmembrane glycoprotein that mediates viral attachment. The G-protein has an ectodomain consisting of a central region of four conserved cysteines, a putative receptor binding site, flanked by two hypervariable regions(6). Several G-protein genotypes have been documented within each subgroup based predominantly on the second hypervariable region(13, 21, 24).

Evolutionary studies of subgroup B strains have described major differences in G-protein length due to alternative termination codon usage, premature stop codons, inframe duplications, deletions and insertions(11, 22). G-proteins of especially subtype B isolates have been identified that are truncated by 30 base pairs due to frame shift mutations(12). The central region remained conserved in all sequences with an absolute conserved region between amino acid positions 164-187(26).

Cell surface glycosaminoglycans (GAGs) are responsible for the majority of RSV attachment to cultured cells leading to infection(4). RSV virions containing the F-protein as sole surface protein bind to GAGs as well as another unidentified molecule suggesting the F-protein may have an auxiliary role as an attachment protein(16).

Variation in neutralizing epitopes in the hypervariable region of the G-protein suggest immune selection of new variants may contribute to generation of HRSV diversity(20). A cold-passaged subtype B mutant containing large deletions spanning most of the coding sequences of the small hydrophobic (SH) and attachment (G) proteins was generated in-vitro. This virus replicated efficiently in Vero cells, but was found to be overattenuated in RSV sero-negative infants and children(7). Deletion of the central conserved domain and cysteine noose was shown not to affect virus growth in vitro or in vivo (in mice)(17), although no record exists of strains that lack this in humans with clinical disease. Augmentation of the Th2
immune response by the G-protein as well as antigenic variation in G makes delta G mutants attractive vaccine candidates(18).

As part of a molecular epidemiology investigation of a nosocomial outbreak of RSV in a ward for premature infants in Kalafong hospital in Pretoria, South Africa in 2006, RSV positive nasopharyngeal aspirates (NPA) from patients in the general paediatric ward were investigated. The HIV-1 seroprevalence among mothers to these infants was 52.6%, suggesting a high level of perinatal exposure(25). Here we describe identification of G-protein deletion mutants that were significantly reduced in size in comparison to prototype controls in children with pneumonia. To investigate this phenomenon these strains were amplified and sequenced with primers that stretch from the start of G- across to the F-protein. In total 209 children were screened that were < 1 year of age and diagnosed with RSV between February 2006 and May 2009 with the immunofluorescence Respiratory Panel 1 IF Assay that detects RSV, PIV1-3, Adenovirus and Influenza A and B (Chemicon, Hampshire, UK) or with the Directigen™ RSV rapid test (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ), at the department Medical Virology, University of Pretoria, Tshwane academic division, National Health Laboratory Service (NHLS) which serves three secondary and tertiary hospitals in the Pretoria region: Kalafong Secondary-, Steve Biko Academic- and the 1-Military Hospital.

RNA was extracted directly from RSV positive NPAs with the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) and subtyped with multiplex nested RT-PCR that distinguish subtype A and B by size(10, 23, 24). Positive specimens resulted in PCR fragments of 950bp for RSV subtype A and 1200bp for subtype B strains in the first round and 700 and 950bp respectively for the nested PCR corresponding with the RSV prototype strain A2 and B1. Figure 1 indicates a diagram of the different primers used.

Two clinical specimens were identified with amplicons that were significantly reduced in size in comparison to the prototype controls. Sequence analysis identified G-protein deletion mutant subtype B strains that lacked most of the ectodomain, including the conserved cysteine noose. These deletion mutant strains as well as one representative strain of each subtype B genotype were selected for full length G-protein sequencing. Furthermore, any specimens that had G-protein subtyping amplicons smaller than the expected size were selected for sequencing as well as a truncated strain previously identified in South Africa AgJ15_99(23). The full length G-protein was amplified with primers: G1-21(22) and F164 (figure1) with the Titan one-step RT-PCR system (Roche diagnostics, Mannheim, Germany)
according to the manufacturer’s recommendations with the following cycle: 50°C for 30min, 94°C for 2min, (94°C for 10sec, 53°C for 30sec, 68°C for 1min) X35 cycles, 68°C for 7min. This was followed by nested PCR with primers: G32B(15) and F1(14) as described before(23). PCR products were analyzed on a 1.5% agarose gel, against a 100-bp molecular weight marker (Roche Diagnostics, Mannheim Germany).

Nucleotide sequencing was carried out using the BigDye version 3.0 Kit on an ABI 3130 sequencer (Applied Biosystems, Foster city, CA) using primers G32B; G604B (5’AAACCAACCATCAAACCCACA3’); F1 and G665R (5’TTTTGGGGCTCTTTTGTTTG3’) (24). Sequence alignments were performed with Clustal X version 1.81(19) and analysed with BioEdit Sequence Alignment Editor version 7.0.4.1(5).

The first specimen with a suspicious G-protein amplicon (SA367859Pt06) was from an 8 month old HIV positive child hospitalized with pneumonia that tested positive for RSV by the RSV rapid antigen kit. This child had symptoms of breathing distress and tachypnoea, very underweight (close to marasmic) and was on continues oxygen treatment. His CD4 count was 1038(27.5%). The percentage of CD4 cells is the best indicator of immunodeficiency in children, although guidelines only exist for infants older than 12 months. The immune system of infants <1 year of age are still immature and HIV infected infants are at serious risk of morbidity and mortality(1). The subtyping multiplex nested PCR resulted in a fragment of approximately 450bp (Figure 2A). Sequencing and blast search analysis identified close homology to the RSV G-protein. PCR amplification and sequencing from the start of G-through to the F-protein and alignment to other RSV strains identified a subtype B strain for which amino acids 101 to 297 (Figure 3) were deleted corresponding to the BA1428/99B (AY333364) reference strain used(21).

One further specimen (SA374801Pt06) was identified that had 3 amplicons of significantly reduced size (Figure 2B lane 4). This specimen was from a 2 month, 13 day old HIV exposed male hospitalized for seven days for gastroenteritis and severe dehydration. He developed pneumonia in the ward and tested positive for RSV five days after admission. The child and mother live in an informal settlement with limited resources and the infant was extremely malnourished upon admission. HIV PCR was performed due to HIV sero-positive status of the mother as part of the antiretroviral treatment programme for prevention of mother to child transmission. Follow up testing for HIV would be carried out up to 18months as per protocol for HIV exposed children. Sequence analysis and BLAST searches confirmed the 200 and 150bp bands to have homology to RSV subgroup B while the larger band was nonspecific.
In both RSV deletion mutant species identified in this patient nearly the entire ectodomain of the G-protein gene was deleted. The smaller of the two RSV-specific amplicons had a deletion from amino acid 31 to 299 while amino acids 87 to 280 were deleted in the larger of the two amplicons (Figure 3). The amplicon from patient 1 and both amplicons from patient 2 lacked the conserved cysteine region. To our knowledge, this is the first time that strains with such extreme deletions in the G glycoprotein have been identified in children with lower respiratory tract infections which shows the flexibility of the G-protein to tolerate significant antigenic changes in nature. No amplicons were visible at the expected position for full length G-proteins and no other co-infections were identified. SA374801Pt06 was negative for Human metapneumovirus, Bocavirus, Parainfluenza viruses 1,2 and 3, Influenza A and B, Adenovirus and Corona viruses- 229E, OC43, HKU1 and NL63 by multiplex real-time PCR(8). SA367859Pt06 was negative for other viruses detected by the IFA but insufficient material was available for additional PCRs. Attempts to culture virus from these specimens was not successful, probably due to low concentrations present and repeat freeze thawing of specimens that had been screened retrospectively.

These findings suggest that despite being attenuated in immunocompetent children, the G-protein might not be necessary for infection and replication in immunosuppressed individuals. These viruses may not be pathogenic or able to replicate in immunocompetent individuals and therefore not detected previously in patients. The F-protein might be sufficient to play the role of auxiliary attachment protein in such cases. It is unclear if the deletion mutants evolved in these children or if they were transmitted. Lazar et al.(9) identified a premature stop codon in the G glycoprotein gene when sequencing strains at three different time points from a patient with severe combined immune deficiency syndrome. The RSV G gene encoded a truncated G glycoprotein lacking 42 carboxy-terminal amino acids but still had the conserved central cysteine noose. The investigators hypothesized that these mutations developed during prolonged infection due to severe immune deficiency in these patients and immunologic pressure as a result of the monthly treatment with intravenous immunoglobulin.

To conclude, data presented here suggests that RSV clinical strains lacking most of the G-protein gene including the central conserved cysteine noose may occur in immunocompromised patients with lower respiratory tract infection. Reduced immune pressure in these patients may allow these strains to utilise other proteins for binding and replication. This may have implications for the utilisation of certain attenuated strains as vaccine candidates in immunocompromised children.
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References


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**Figure 1** Diagram of primers used. G32B, G267A and F164: 1st round multiplex RT-PCR; G52B, G283A and F1: Nested multiplex PCR for subtyping. G1-21 and F164: 1st round RT-PCR; G32B and F1: Nested PCR for full length G-protein amplification. G32B, G604B, F1 and G665R: Sequencing primers.

**Figure 2** G protein specific subtyping multiplex nested RT-PCR. (A) Amplicon from Patient 1 (P1) (SA367859Pt06) next to a negative control and the prototype subtype A and B-positive control strains. (B) Multiplex nested PCR products; Lane 1-5: Clinical specimens. Lane 6: Negative control. Lane 7: Positive control RSV A. Lane 8: Positive control RSV B. Lane 4: Deletion mutant 2 (SA374801Pt06).

**Figure 3** Complete amino acid alignment of the three deletion mutants in comparison to the full G-protein subtype B sequences of each genotype. The genotype name is indicated after the strain name. Accession numbers: HQ711840-HQ711842 (Deletion mutant strains SA367859Pt06; SA374801Pt06a; SA374801Pt06b); AF065250 (GB1); AF065251 (GB2); JF704217 (GB3); JF704214 (GB4); JF704213 (SAB1); JF704218 (SAB2); JF704215 (Truncated SAB3); JF704216 (SAB3); AY333364 (BA); JF704219 (South African BA).
Venter et al. Figure 1

- G-protein
  - Transmembrane domain
  - 1st hypervariable region
  - Central Cysteine Domain
  - 2nd hypervariable region

- F-protein
  - Subtyping of subtype B
  - Full length subtype B G protein