ORIGINAL ARTICLE

# Detection and subgrouping of respiratory syncytial virus directly from nasopharyngeal aspirates

*Reena Ghildyal[1], Geoff Hogg[2], John Mills[1] and Jayesh Meanger[1]*

[1]Children's Virology Research Unit, Macfarlane Burnet Centre for Medical Research, Fairfield, and [2]Department of Microbiology and Infectious Disease, Royal Children's Hospital, Flemington, Victoria, Australia

**Objective:** To develop a reverse transcription–polymerase chain reaction (RT-PCR)-based assay to identify the subgroup of the infecting respiratory syncytial virus (RSV) strain directly from nasopharyngeal aspirates (NPAs).

**Methods:** A total of 154 NPAs which were positive for RSV antigen by direct immunofluorescence were subjected to RT-PCR. The primers used were designed to give products for subgroup A and B which were of different sizes and easily visualized on agarose electrophoresis. The PCR products were further analyzed by restriction analysis using enzymes which were unique or rare cutters within the PCR amplimer.

**Results:** It was possible to confirm RSV infection in 70% of the NPA samples studied. Of these, 92.6% belonged to the A group, and only 7.4% to the B group. Within the A group, six subgroups were identified using restriction analysis, while all B-group samples were identical to the prototype B strain, 18537.

**Conclusion:** RT-PCR performed on RNA isolated directly from NPAs provides a quick, easy-to-use, reasonably sensitive method to identify and group the infecting RSV strain.

**Key words:** RSV subgrouping, RT-PCR, nasopharyngeal aspirates

Respiratory syncytial virus (RSV) is the major viral pathogen causing severe lower respiratory tract infections in infants, and it also causes significant morbidity amongst the elderly and in immunocompromised adults. Annual epidemics occur in late autumn and winter in temperate regions or during the rainy season in tropical countries [1]. It has been known for some time that there are two antigenic variants of RSV, designated A and B [2]. Group A and B often co-circulate during epidemics, A usually accounting for 70–80% of cases and causing more severe disease than B [3].

RSV infections are currently diagnosed by recovery of virus in cell culture, or by detection of viral antigens in nasopharyngeal secretions by immunofluorescence or enzyme immunoassays [4,5]. In this report, we describe a rapid method of detection of RSV which also differentiates between the two groups, and which can be used for subgrouping. The method is based on reverse transcription and polymerase chain reaction (RT-PCR) amplification of virion mRNA isolated from nasopharyngeal aspirates [6]. In addition to providing rapid detection, this method provides group information which would be useful for treatment, epidemiologic and transmission studies, and the development of an RSV vaccine.

Corresponding author and reprint requests:

John Mills, Children's Virology Research Unit, Macfarlane Burnet Centre for Medical Research, Yarra Bend Road, PO Box 254, Fairfield, Victoria 3078, Australia

Tel: 613 9282 2123 Fax: 613 9282 2100

e-mail: mills@burnet.mbcmr.unimelb.edu.au

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## MATERIALS AND METHODS

### Virus strains

RSV A2 and 18537 strains (the prototype A and B strains respectively, a kind gift from Dr. Paul Young, SASVRC, Brisbane, Qld) were grown in HEp-2 cells. At 18 h postinfection, the cells were harvested and RNA extracted using the guanidinium thiocyanate method [7]. This RNA was used in all experiments as

a positive control. Uninfected HEp-2 cells were treated in exactly the same way and used as a negative control in the RT-PCR.

### Patient samples

Nasopharyngeal aspirates (NPAs) containing RSV antigen according to immunofluorescence were obtained from the Royal Children's Hospital, Melbourne. All the specimens were from children seen at the hospital during the winter (May to September) of 1995 and had been stored at −20°C in virus transfer medium. In all, 154 specimens were screened by our method.

### Extraction of RNA

RNA was extracted directly from the NPAs by the guanidinium thiocyanate (GT) method described previously [7]. Briefly, 0.5 mL of NPA sample was diluted to 5 mL with saline, and cells were isolated by centrifugation at 900*g* for 10 min after mixing well. The cells were suspended in 0.5 mL of GT solution containing 4 M guanidinium thiocyanate in 25 mM sodium citrate (pH 7) containing 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. The lysate was extracted once with phenol/chloroform (1 : 1, v/v) and RNA was precipitated with isopropanol. The pellets were washed once with chilled 70% ethanol and suspended in 10 μL of water.

### Oligonucleotides

The oligonucleotides used for RT-PCR were same as those described by Sullender et al. [8]. The 3′ oligonucleotide BH2 was complementary to bases 164–186 in the F protein mRNA of CH18537 and had one mismatch to the A2 F protein mRNA sequence (GCCCTAGGGCGTTATAACACTGGTATACCAA CC). The two 5′ oligonucleotides A and B used for PCR conferred group specificity to the assay. The A-group primer corresponds to bases 247–267 in the A2 G glycoprotein mRNA (TGCCCTAGGTA GATGCAACAAGCCAGATCAAG) and the B-group primer corresponds to bases 10–30 in the 8/60 G glycoprotein mRNA (TGCCCTAGGTAGCAACC ATGTCCAAACACAAG). Both primers also contain a *Bam*H1 restriction endonuclease recognition sequence at the 5′ end (underlined in the sequences above). The expected size of the PCR products were 900 bp for group A and 1100 bp for group B, as described by Sullender et al. [8].

### Reverse transcription

RNA prepared from nasopharyngeal aspirates was used as a template for cDNA synthesis. A 20-μL reaction volume contained 5 μL of RNA (approximately 1 μg), 0.1 u of avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp.), 10 u of RNasin (Promega Corp.), 50 pmol of BH2, 2 mM concentrations of dNTPs (dGTP, dATP, dTTP and dCTP), and 10 mM DTT in reaction buffer (× 5 buffer supplied by the manufacturer, containing 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol (DTT) and 2.5 mM spermidine). Synthesis was performed at 37°C for 45 min and enzyme was inactivated at 65°C for 5 min.

### DNA amplification

Of the above cDNA preparation, 5 μL was used for PCR amplification. A 50-μL reaction volume contained 50 pmol of all three primers (BH2, the non-specific primer, and both the group A and group B specific primers), 0.5 u of DNA polymerase (from *Thermus icelandicus*, manufactured by Advanced Biotechnologies Ltd, USA), 2.5 mM $MgCl_2$, and 2 mM dNTPs, in reaction buffer (× 10 buffer supplied by the manufacturer, containing 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl (pH 9), 0.1% Tween). The reaction mix was heated at 94°C for 2 min in a Perkin Elmer 9600 thermocycler for one cycle and then subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, terminating with final extension for 7 min at 72°C before being cooled to 4°C. From each sample, a 10-μL volume of each sample was analyzed on a 1% agarose gel in Tris-borate EDTA buffer, and visualized by ethidium bromide staining.

### Restriction analysis

The double-stranded PCR products (10 μL) were analyzed by agarose electrophoresis before and after restriction endonuclease digestion to study the genetic variation within each group, as described by Sullender et al. [8]. These enzymes are readily available commercially and are unique or rare cutters within RSV cDNA from the F and G genes.

## RESULTS

We analyzed 154 NPAs by the RT-PCR method described above, and 70% (108; 95% confidence interval, 62–77%) of them yielded a PCR product of the expected size by gel electrophoresis (Figure 1a). Of these, 100 (92.5%) were group A, the remainder being group B. There was no evidence of dual infection. Analysis of the PCR amplimers by restriction digestion and gel electrophoresis revealed six different subgroups within group A, one of which was identical to the A2 strain (Figure 1b; Table 1). All eight group B specimens were identical to the reference B group (18537) in these restriction digests.

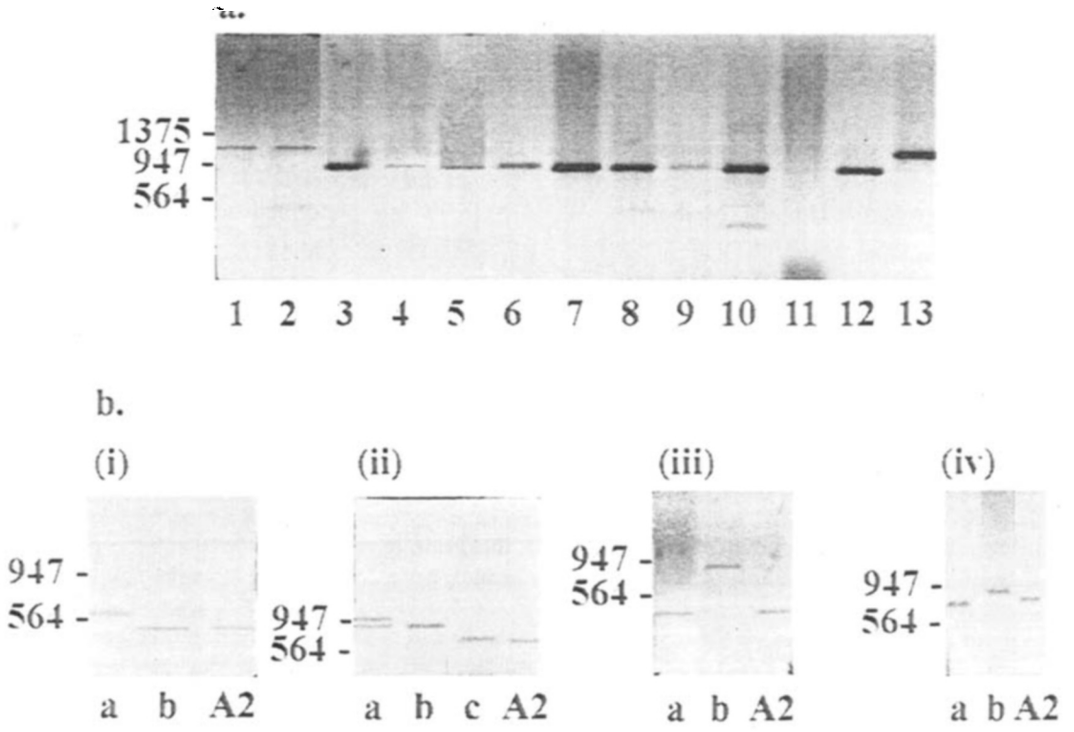


**Figure 1** Analysis of RT-PCR amplimers of RSV mRNA by gel electrophoresis, with or without restriction digestion. (a) RNA was extracted from patient NPAs and RT-PCR performed as described in the text. The products were analyzed on 1% agarose gel. Lanes 1–10: patient samples. Lanes 1 and 2: samples identified as group B. Lanes 3–10: samples identified as group A. Lane 11: RT-PCR of RNA from mock infected HEp2 cells. Lanes 12 and 13: RT-PCR of RNA from HEp2 cells infected with A2 and 18537 strains of RSV (the reference A and B strains), respectively. Numbers on the left indicate molecular size (bp). (b) Restriction enzyme digestion patterns obtained from group A samples. RT-PCR products were digested with various restriction enzymes and patterns analyzed on 1% agarose gels. Lanes a, b, and c are patterns obtained from samples. Lanes A2 are patterns obtained from strain A2. (i) *Pst*1 digestion; (ii) *Alu*1 digestion; (iii) *Rsa*1 digestion; (iv) *Hind*II digestion. Numbers on the left indicate molecular size (bp).

**Table 1** Subgrouping of RSV group A on the basis of restriction patterns

| | *Pst*I | | *Rsa*I | | *Alu*I | | | *Hind*II | |
|---|---|---|---|---|---|---|---|---|---|
| Subgroup | 1 | 3 | 1 | 2 | 1 | 2 | 3 | 1 | 2 |
| a | + | | + | | + | | | + | |
| b | + | | + | | + | | | | + |
| c | + | | + | | | | + | + | |
| d | + | | + | | | + | | + | |
| e | + | | | + | + | | | | + |
| f | | + | + | | | + | | + | |

1, Pattern identical to that obtained from A2 strain; 2, Sample was resistant to digestion by this enzyme; 3, Pattern obtained was different from that obtained from A2 strain.

## DISCUSSION

This study shows that it is technically possible to detect RSV mRNA directly in frozen NPAs by an RT-PCR technique which also yields information about the serogroup (A or B) of the infecting strain. Although the sensitivity of the technique was not ideal (70%), the NPA samples had been frozen at –20 °C for varying periods of time, without RNase inhibitors, which would probably result in substantial RNA degradation.

Consistent with previous studies [8,9], the majority of infecting strains in the 1995 season belonged to group A. There is only one recorded instance of both groups occurring to the same extent [10]. The different patterns obtained by restriction digestion show that there is genetic variability within the groups prevalent in one season. Antigenic variability within a group of RSV is well documented [11] and there is a recent report on genetic variation among the A-group RSV isolated at various times in different locations [12]. Sullender et al. reported the presence of two genetically different isolates within an antigenically identical group

of isolates from a single season. To our knowledge, this is the first time that multiple genetically distinct isolates have been demonstrated within an infecting group in one season.

Traditionally, diagnosis of RSV infection has been made by observation of typical cytopathic effects in mammalian cell culture, although electron microscopy might be considered in certain cases [4]. Culture requires viable virus in the NPAs, and, as RSV is a slow-growing virus, positive identification can take up to 12 days, especially in the case of B-group virus. In recent years, detection of RSV antigens in NPAs by enzyme immunoassay and detection by immunofluorescence techniques have come into use as alternative methods of RSV diagnosis [4,5]. These have appreciably reduced the time required for diagnosis, although there is some loss in sensitivity as compared with culture. PCR technology has also been developed as means of rapid, accurate diagnosis of numerous infectious agents [6,8,13–16] with the same level of success. Paton et al. [6] have described a method for diagnosis of RSV infection based on RT-PCR which could be applied to NPAs directly. The PCR product so obtained was only 243 bp, and was occasionally difficult to visualize in agarose gels. In addition, no grouping information was obtained. Sullender et al. [8] have described an RT-PCR-based method which can reliably differentiate groups of RSV using RNA isolated from infected cell lysates. RT-PCR performed on nasal aspirates followed by hybridization by group-specific probes in a DNA enzyme immunoassay has been used recently [14] to increase the level of detection over previously described RT-PCR methods, although this is much less practical than simple RT-PCR for clinical use.

The method we describe here is relatively simple, reasonably sensitive, and, in contrast to other methods, provides full information about virus groups, which is useful for research studies. This method also has potential for use as a routine clinical test in a molecular diagnostic laboratory once it has been optimized and is as sensitive as the currently used techniques.

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**References**

1. McIntosh K, Chanock RM. Respiratory syncytial virus. In Fields DN, Knipe DM, eds. Virology. New York: Raven Press, 1990: 1045–72.
2. Mufson MA, Orvell C, Rafnar B, Norrby E. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol 1985; 66: 2111–24.
3. Hendry RM, Burns JC, Walsh EE, et al. Strain specific serum antibody responses in infants undergoing primary infection with respiratory syncytial virus. J Infect Dis 1988; 157: 640–7.
4. Welliver RC. Detection, pathogenesis and therapy of respiratory syncytial virus infections. Clin Microbiol Rev 1988; 1: 27–39.
5. Takimoto S, Grandien M, Ishida MA, et al. Comparison of enzyme–linked immunosorbent assay, indirect immunofluorescence assay, and virus isolation for detection of respiratory viruses in nasopharyngeal secretions. J Clin Microbiol 1991; 29: 470–4.
6. Paton AW, Paton JC, Lawrence AJ, Goldwater PN, Harris RJ. Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification. J Clin Microbiol 1992; 30: 901–4.
7. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987; 162: 156–9.
8. Sullender WM, Lirong Sun, Anderson L. Analysis of respiratory syncytial virus genetic variability with amplified cDNAs. J Clin Microbiol 1993; 31: 1224–31.
9. Mlinaric-Galinovic G, Chonmaitree T, Cane PA, Pringle CR, Ogra PL. Antigenic diversity of respiratory syncytial virus subgroup B strains circulating during a community outbreak of infection. J Med Virol 1994; 42: 380–4.
10. Hierholzer JC, Tannock GA, Hierholzer CM, Coombs RA, Kennett ML, Phillips PA, Gust ID. Subgrouping of respiratory syncytial virus strains from Australia and Papua New Guinea by biological and antigenic characteristics. Arch Virol 1994; 136: 133–47.
11. Gilchrist S, Torok TJ, Gary HE Jr, Alexander JP, Anderson LJ. National surveillance for respiratory syncytial virus, United States, 1985–1990. J Infect Dis 1994; 170: 986–90.
12. Sanz MC, Kew OM, Anderson LJ. Genetic heterogeneity of the attachment glycoprotein G among group A respiratory syncytial viruses. Virus Res 1994, 33: 203–17.
13. van Milaan AJ, Sprenger MJW, Rothbarth PhH, Brandenburg AH, Masurel N, Claas ECJ. Detection of respiratory syncytial virus by RNA–polymerase chain reaction and differentiation of subgroups with oligonucleotide probes. J Med Virol 1994; 44: 80–7.
14. Freymuth F., Eugene G, Vabret A, et al. Detection of respiratory syncytial virus by reverse transcription-PCR and hybridisation with a DNA enzyme immunoassay. J Clin Microbiol 1995; 33: 3352–5.
15. Gilbert LL, Dakhama A, Bone BM, Thomas EE, Hegele RG. Diagnosis of viral respiratory tract infections in children by using a reverse transcription-PCR panel. J Clin Microbiol 1996; 34: 140–3.
16. Gottschalk J, Zbinden R, Kaempf L, Heinzer I. Discrimination of respiratory syncytial virus subgroups A and B by reverse transcription-PCR. J Clin Microbiol 1996; 34: 41–3.