

## Use of induced sputum for the diagnosis of influenza and infections in asthma: a comparison of diagnostic techniques

Jodie L. Simpson<sup>a</sup>, Ivana Moric<sup>b</sup>, Peter A.B. Wark<sup>a</sup>, Sebastian L. Johnston<sup>c</sup>, Peter G. Gibson<sup>a,\*</sup>

<sup>a</sup> Department of Respiratory and Sleep Medicine, John Hunter Hospital, Locked Bag 1, Hunter Region Mail Centre, Newcastle, New South Wales 2310, Australia

<sup>b</sup> Institute for Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, 11000 Belgrade, Yugoslavia

<sup>c</sup> Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College of Science, Technology and Medicine, Norfolk Place, London W2 1PG, United Kingdom

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### Abstract

**Background:** Influenza (Flu) and respiratory syncytial virus (RSV) are important viral pathogens that cause lower respiratory tract infections and severe exacerbations of asthma. Molecular biological techniques are permitting a rapid and accurate diagnosis of infections caused by respiratory pathogens, and have typically been applied to upper respiratory samples. Sputum induction provides an opportunity to directly sample secretions from the lower respiratory tract. **Objectives/study design:** To determine the role of induced sputum reverse-transcription polymerase chain reaction (RT-PCR) in the detection of respiratory pathogens and compare this with detection using serology and immunofluorescent antigen (IFA) testing, we recruited 49 adults from emergency room with exacerbations of asthma. After a medical assessment and spirometry, sputum was induced using ultrasonically nebulised normal saline. Sputum was assayed using IFA and RT-PCR for flu and RSV. Flu serology was performed acutely and at convalescence, 4–5 weeks later. **Results:** Influenza A or B was detected in 24% of the samples by PCR, significantly more than the nine cases detected using serology and the one case using IFA ( $P < 0.05$ ). RSV was detected in 37% of samples using PCR and 20% by IFA ( $P < 0.05$ ). **Conclusion:** The combination of induced sputum and RT-PCR provides a useful means of detecting respiratory infection. The technique is safe in both adults and children, and RT-PCR is more sensitive than conventional serology and IFA. The improved sensitivity of induced sputum RT-PCR also permits a more rapid diagnosis and the opportunity of early administration of effective treatments.

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**Keywords:** Influenza; Respiratory syncytial virus; Induced sputum; Polymerase chain reaction

**Abbreviations:** Flu, influenza; RSV, respiratory syncytial virus; RT-PCR, reverse-transcription polymerase chain reaction; IFA, immunofluorescence antigen; PCP, *Pneumocystis carinii*; PEF, peak expiratory flow; FEV<sub>1</sub>, forced expiratory volume in 1 s.

\* Corresponding author. Tel.: +61-2-492-13470; fax: +61-2-492-13469

E-mail address: [mdpgg@mail.newcastle.edu.au](mailto:mdpgg@mail.newcastle.edu.au) (P.G. Gibson).

## 1. Introduction

Viral respiratory tract infections are the main cause of asthma exacerbations in both community (Nicholson et al., 1993; Johnston et al., 1995) and hospital settings (Wark et al., 2002; Teichtahl et al., 1997). The most common viruses isolated are rhinovirus, influenza (Flu) and respiratory syncytial virus (RSV) (Nicholson et al., 1993; Johnston et al., 1995; Wark et al., 2002; Teichtahl et al., 1997). Exacerbations of asthma are a major burden of illness to the community, resulting in hospitalisations and school absenteeism. Combinations of nasopharyngeal aspirate and serology (Nicholson et al., 1993; Johnston et al., 1995; Teichtahl et al., 1997) are typically used to diagnose viral respiratory infections, although these are seldom applied to adults and children with exacerbations of asthma. RSV and flu predominantly infect the lower respiratory tract, and in the case of RSV, samples obtained from the lower respiratory tract contain greater quantities of virus and are more sensitive than nasal wash/throat swab (Englund et al., 1996). Induced sputum has emerged as an important sampling technique for the assessment of lower airway secretions and is less invasive than bronchoscopy (Gibson et al., 2000). The application of molecular biological techniques has permitted rapid and more accurate diagnosis of infections caused by respiratory viruses (Turner, 1990) and other organisms (Sing et al., 2000; Mathis Am Weber et al., 1997; Leibovitz et al., 1995; Khan et al., 1999; Shimotmoto et al., 1995) in other settings.

We therefore hypothesised that the application of reverse-transcription polymerase chain reaction (RT-PCR) viral diagnostics to induced sputum would perform better than immunofluorescent antigen (IFA) detection and serological techniques for the detection of flu and RSV infections. Our focus was on the performance characteristics of rapidly available tests, and in this report we compare RT-PCR, IFA and serology for the diagnosis of these viral respiratory infections in subjects with acute asthma.

## 2. Methods

### 2.1. Subjects

We included adults ( $n = 49$ ), aged 16–74 years (18 male), who presented to the emergency room of John Hunter Hospital with an acute exacerbation of asthma and who had sputum induced for analysis. Asthma was defined at the initial interview by a respiratory physician using American Thoracic Society criteria based upon a previous physician diagnosis, a compatible history, together with either bronchial hyperresponsiveness (BHR;  $PD_{15}$  saline  $< 15$  ml) or an improvement in  $FEV_1$  of  $> 15\%$  after salbutamol 200  $\mu$ g. An acute exacerbation of asthma was based upon deterioration in symptoms of wheeze, breathlessness or cough, increase in use of short-acting  $\beta_2$  agonist for symptom relief or a deterioration in self-monitored peak expiratory flow (PEF) leading to emergency room presentation. Subjects were excluded if they did not fulfil the inclusion criteria, were on regular oral corticosteroids for their asthma, had a co-existing physician diagnosis of chronic obstructive pulmonary disease, an infiltrate on chest radiograph suggesting pneumonia, a complication of asthma or any other contraindication to sputum induction. We excluded ten subjects who were unable to produce an adequate sputum sample.

### 2.2. Design

Following medical assessment and initial treatment, spirometry was performed. Subjects then received salbutamol, and sputum was induced by inhalation of 0.9% saline (Norzilla et al., 2000; Wark et al., 2001; Twaddell et al., 1996). Venepuncture was performed for acute serology and sputum sent to the laboratory for processing. Convalescent serology was collected at a follow-up assessment 4–5 weeks later.

### 2.3. Sputum induction

Sputum induction was performed on subjects with a  $FEV_1$  or PEF greater than 25% of the predicted value after pre-treatment with salbuta-

mol 200 µg, delivered by a pressurised metered dose inhaler and volumatic spacer (Allen and Hanbury's, Melbourne, Australia) (Norzilla et al., 2000; Wark et al., 2001; Twaddell et al., 1996). All subjects received supplemental oxygen during sputum induction. Induced sputum was obtained using sterile normal saline (0.9%) delivered from a DeVilbiss 2000 ultrasonic nebuliser (Oregon Pike, PA) with 23 cm corrugated tubing and a Hans Rudolf 2700 two-way non-rebreathing valve box (Hans Rudolf Inc, Kansas City, KS), rubber mouthpiece and nose clip. Saline was nebulised for 30 s, 1 min and then for 2 min period, until an adequate sample was obtained. Spirometry was performed 1 min after each nebulisation period to monitor the development of airflow obstruction, and supplemental salbutamol was administered if there was a fall of 20% or greater in spirometry from baseline. Sputum induction was ceased when an adequate sample was obtained, if spirometry fell below 20% of baseline and did not recover within 10 min, at the subject's request or at the investigator's discretion. Following these careful guidelines, there was no serious adverse reactions to the nebulised saline (0.9%). The greatest fall in FEV<sub>1</sub> was 22% from baseline and only eight subjects experienced a fall in FEV<sub>1</sub> of 15% or more.

#### 2.4. Microbiological methods

RSV antigen was detected using an enzyme-linked fluorescent immunoassay (ELFA, Vidas RSV, Bio Merieux Ltd., Lyon, France), according to manufacturer's instructions using a VIDAS (Vitek ImmunoDiagnostic Assay System, Lyon, France) instrument. Briefly, samples were added to bovine anti-RSV antibody-coated sample receptacles and antigens allowed binding. Unbound sample was removed and receptacles were blocked using guinea pig anti-RSV serum. After washing, goat anti-guinea pig IgG conjugated to alkaline phosphatase was added. Excess conjugate was removed and a fluorescent substrate (4-methylumbelliferyl phosphate) was added. Any enzyme remaining on the receptacle walls catalysed the conversion of the substrate to the fluorescent produce (4-methylubelliferone) which was mea-

sured using an optical scanner within the instrument. Results were reported as positive or negative. Negative results were confirmed using Bartels Viral Respiratory Screening and Identification Kit (Bartels Inc., Issaquah, WA) as outlined below, as false-negative results can occur due to poor sample collection.

Respiratory viruses were assessed in sputum using an indirect immunofluorescence assay according to Bartels Kit manufacturer's instructions. Briefly, sputum was aliquoted, washed and cells were spotted onto a 10-well slide. Slides were fixed, and antibody identification reagent and anti-viral antibody were added to the appropriate wells on patient and antigen control slides, respectively. Non-immune mouse antibody was then added to a separate well on all slides and slides incubated for 30 min at 37 °C. After washing, anti-mouse FITC conjugate was added to all wells and slides were incubated for a further 30 min at 37 °C. Slides were washed, mounted using buffered glycerol and observed using a fluorescent microscope within 24 h. The Bartels kit reagents contain antibodies directed against adenovirus, influenza A, influenza B, parainfluenza types 1, 2 and 3 and RSV. Detection methods for rhinovirus or coronaviruses were not evaluated, as there was no rapid detection method available, other than PCR.

#### 2.5. Serology

Venous blood was collected at visit 1 and 2 for the determination of acute and convalescent antibody titers. Blood was centrifuged and serum stored at 4 °C until tested. The presence of recent infection was defined serologically by a fourfold rise in titres from acute-to-convalescent serology for influenza A or B. A compliment fixation test was used to detect antibodies to influenza A and influenza B. Briefly, serum was diluted, and then serum and control dilutions were deactivated for 30 min at 56 °C. Samples were added to the test plate and microdiluted (1:4–1:256). Serum and antigen controls were also added to each test plate. Diluted compliment was added to each well prior to an overnight incubation at 36 °C. The end point was determined at 75% of RBC settling and titres reported.

## 2.6. RT-PCR

A whole sputum plug was selected and aliquoted immediately after collection and stored at  $-70^{\circ}\text{C}$  for virus PCR. Sputum RNA was extracted using Trizol (Gibco BRL, Life Technologies, NY) (Chauhan and Johnston, 2000). Briefly, after shaking the plug overnight at  $4^{\circ}\text{C}$  in 5 ml RNase-free water, 50  $\mu\text{l}$  of the diluted sputum was mixed with Trizol reagent (450  $\mu\text{l}$ ), chloroform (50  $\mu\text{l}$ ) was added and the sample was mixed and centrifuged at 12 000 rpm for 15 min at  $4^{\circ}\text{C}$ . The upper phase was removed into ice-cold isopropanol (800  $\mu\text{l}$ ) and stored overnight at  $-20^{\circ}\text{C}$ . Samples were thawed and centrifuged at 13 000 rpm for 20 min at  $10^{\circ}\text{C}$  before washing the pellet in 70% ethanol. Dried RNA pellets were resuspended in 1  $\mu\text{l}$  (33.7U) RNAGuard (Pharmacia Biotech, Uppsala, Sweden) and 20  $\mu\text{l}$  of water.

The diluted RNA was combined with 2.5  $\mu\text{l}$  (1.25  $\mu\text{g}$ ) of random hexamers (Promega Corporation, Madison, WI) and water (4  $\mu\text{l}$ ) and heated to  $70^{\circ}\text{C}$  for 10 min before being placed on ice. To this mixture were added 2  $\mu\text{l}$  of SuperScript (200U, Life Technologies, NY), 10  $\mu\text{l}$  of  $5\times$  buffer, 5  $\mu\text{l}$  of 1 mM dithiothreitol (both supplied with SuperScript), 1.25  $\mu\text{l}$  (62.5  $\mu\text{M}$ ) deoxyribonucleoside triphosphates (dNTPs, Promega Corporation) and the samples were incubated for 1 h at  $37^{\circ}\text{C}$ . The cDNA (50  $\mu\text{l}$ ) was then stored at  $-70^{\circ}\text{C}$  until PCR was performed.

Sputum PCR was performed for influenza A and B (Ellis et al., 1997) and RSV (O'Donnell et al., 1998). RT-PCRs detect either genomic or mRNA specific for each virus. The primary cDNA (2  $\mu\text{l}$ ) was combined with 5  $\mu\text{l}$  of PCR buffer, 5  $\mu\text{l}$  of magnesium chloride, 0.5  $\mu\text{l}$  (2.5  $\mu\text{M}$ ) dNTPs, 0.5  $\mu\text{l}$  of each primer, 0.5  $\mu\text{l}$  of Taq polymerase (Promega Corporation) and 35  $\mu\text{l}$  of water. The second round of PCR was prepared as above replacing the primary cDNA with 2  $\mu\text{l}$  of product from the first round. All primers used for each virus PCR are listed in Table 1. PCR products were visualised using agarose gel electrophoresis with a DNA ladder to determine fragment size. Internal positive and negative controls for PCR were used at the time of testing.

Table 1  
Primers used for viral PCR

<i>RSV primers</i>	
<i>First round</i> (product size 360 bp)	<i>Second round</i> (product size 259 bp)
ATG TCA CGA AGG AAT	GAG GTC ATT GCT TAA
CCT TGC	ATG G
TAG CTC TTC ATT GTC	GCA ACA CAT GCT GAT
CCT CAG	TGT
<i>Influenza primers</i>	
<i>First round</i>	<i>Second round</i>
<i>AH1</i> (product size 1015 bp)	<i>AH1</i> (product size 944 bp)
CAG ATC CAG ACA CAA	ATA GGC TAC CAT GCG
TAT GT	AAC AA
AAA CCG GCA ATG GCT	CTT AGT CCT GTA ACC
CCA AA	ATC CT
<i>AH3</i> (product size 883 bp)	<i>AH3</i> (product size 591 bp)
CAG ATT GAA GTG ACT	AGC AAA GCT TTC AGC
AAT GC	AAC TG
GTT TCT CTG GTA CAT	GCT TCC ATT TGG AGT
TCC GC	GAT GC
<i>B</i> (product size 900 bp)	<i>B</i> (product size 767 bp)
GTG ACT GGT GTG ATA	CAT TTT GCA AAT CTC
CCA CT	AAA GG
TGT TTT CAC CCA TAT	TGG AGG CAA TCT GCT
TGG GC	TCA CC

## 2.7. Statistical methods

Statistical analysis was carried out using STATA (Stata Corporation, College Station, TX). Differences in proportions between groups were analysed by Fisher's exact test or  $\chi^2$ . Significance was accepted when  $P$  was  $<0.05$ .

## 3. Results

Induced sputum samples were collected from 49 acute asthmatic subjects. Influenza A or B was detected by PCR in 12 (24%) samples, by IFA in one (2%) sample and by serology in nine (18.3%) samples ( $P < 0.05$ , PCR and serology vs. IFA—Fig. 1). Comparing influenza IFA with PCR resulted in a sensitivity of 8.3% and a specificity of 100%. Only one sample was both PCR and IFA positive, while a further 11 samples produced a positive PCR result with a negative IFA. Serology

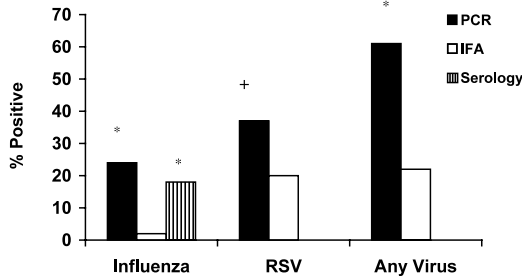


Fig. 1. Respiratory pathogen-positive (%) samples for each detection method. \* $P < 0.05$  versus IFA. + $P = 0.07$  versus IFA.

compared more favourably with PCR with a sensitivity of 81.82% and a specificity of 100% (Table 2). Two samples were PCR positive but negative for both IFA and serology. There were nine samples that were IFA negative but serology positive. No samples were positive for IFA but negative for serology.

RSV was detected by PCR in 18 (37%) samples and ten (20%) by IFA ( $P = 0.07$ ). The assay specificity of IFA was 100% and sensitivity was 58.8% (Table 2). One subject returned a positive PCR result for both influenza A and RSV but negative serology and IFA for all viruses.

Using PCR, either flu or RSV could be detected in 61% of samples whereas detection using IFA resulted in only 22% of samples tested being positive ( $P < 0.05$ ).

#### 4. Discussion

This study demonstrates that sputum induction can be used to obtain lower respiratory secretions for viral diagnosis in acute asthma and describes the performance characteristics of PCR, IFA and serology. PCR gave the highest positivity rate for both flu and RSV. Serology performed reasonably well for flu diagnosis, whereas IFA had had only one positive but was less sensitive than PCR for both flu and RSV detection. The results indicate that the combination of induced sputum and RT-PCR is a useful means to detect viral infection of the lower respiratory tract in asthma.

We studied a population with relatively severe exacerbations of asthma who presented to hospital for treatment. The diagnosis of infection was based on the ability to detect the organism by a combination of serology, DFA or PCR on induced sputum. In this context viral culture is less sensitive in the detection of viruses, while PCR is both sensitive and specific (Liolios et al., 2001) and validated in asthma exacerbations (Nicholson et al., 1993; Johnston et al., 1995).

Clinical studies indicate that most patients with a severe exacerbation of asthma give a history suggestive of viral infection as the precipitant (Nicholson et al., 1993; Johnston et al., 1995; Sokhandan et al., 1995). The use of conventional viral diagnostic techniques such as IFA, serology and viral culture results in a low confirmation rate

Table 2  
Sensitivity and specificity of PCR, immunofluorescent and serologic detection methods in induced sputum

	PCR positive (n)	PCR negative (n)	Sensitivity (%)	Specificity (%)
<i>Flu A/B serology</i>				
Positive	9	0	81.82	100
Negative	3	37	–	–
<i>Flu A/B IFA</i>				
Positive	1	0	8.33	100
Negative	11	37	–	–
<i>RSV IFA</i>				
Positive	10	0	58.82	100
Negative	8	31	–	–

IFA, immunofluorescent antigen (see Section 2 for details).

for viral infection in acute asthma (Teichtahl et al., 1997; Sokhandan et al., 1995). Viral diagnosis using RT-PCR is an emerging technique that is rapid and has the advantage of being both sensitive and specific in the diagnosis of viral infections. Recent comparative studies show that RT-PCR is at least as sensitive as viral culture for the detection of influenza (Liolios et al., 2001; Kehl et al., 2001; Vabret et al., 2000; Wallace et al., 1999) and RSV (Kehl et al., 2001; Walsh et al., 2001) infections. A potential limitation of the present study is that viral culture was not used as the gold standard comparison. However it is increasingly recognised that RT-PCR can detect more positive respiratory pathogens than culture methods (Liolios et al., 2001; Kehl et al., 2001), giving it the best sensitivity of the available diagnostic techniques. Serology was added to validate the PCR results for flu. IFA has good specificity when compared with culture, and gives rapid results. We are therefore unlikely to have missed samples that would have been positive by viral culture. Furthermore, our primary aim was to compare the performance of the available rapid tests, IFA and PCR, in samples of induced sputum.

Prior studies have been conducted primarily using nasal samples, throat swabs, or spontaneous sputum samples. When these samples were compared, spontaneous sputum gave the best predictive value for influenza diagnosis, performing significantly better than nasopharyngeal swab and throat swab, and as good as nasal aspirate (Cavalcicuc et al., 1999). Similarly, lower respiratory tract samples were more sensitive than nasal wash/throat swab for RSV diagnosis (Englund et al., 1996). A key factor in obtaining a good diagnostic success rate is sample quality, with samples containing higher cell numbers having a significantly better influenza detection rate than samples with low cellularity (Noyola et al., 2000). Sputum induction improves the quality and reproducibility of samples. An immediate limitation when applying these results to people with asthma is the ability to obtain adequate sputum from the majority of subjects. Spontaneous sputum is limited by its unpredictable availability and quality. A prior study used nasopharyngeal swabs for

viral diagnosis and induced sputum to examine the inflammatory reaction in the lower airway (Pizzichini et al., 1998). Sputum can be induced using hypertonic saline inhalation, and induced sputum has an established role in the diagnosis of respiratory infection due to tuberculosis and *Pneumocystis carinii* (PCP). Hypertonic saline is a potent stimulus for bronchoconstriction (Anderson et al., 1983) and as such is contraindicated in acute asthma. We have modified the technique to use normal saline delivered via ultrasonic nebuliser for sputum induction in acute asthma (Twaddell et al., 1996). This modified sputum induction technique has a good success rate for lower respiratory samples (Pizzichini et al., 1998) and is safe in both adults (Wark et al., 2001) and children over the age of 6 years (Norzilla et al., 2000; Twaddell et al., 1996) with acute asthma. We now extend these observations to demonstrate that induced sputum samples obtained from adults presenting with acute asthma have a good yield for the diagnosis of viral lower respiratory infection.

Antigen detection and PCR are both available as rapid diagnostic methods for the detection of respiratory viral infection. With induced sputum, the overall detection rate for PCR was considerably better than rapid antigen detection. This suggests opportunities for more widespread use of sputum PCR in viral diagnostics.

In conclusion, induced sputum combined with RT-PCR is more sensitive than both serology and IFA in the detection of flu and RSV infection in subjects with acute asthma. This technique also provides results more rapidly than both cell culture and serology. Combining this with the recent advances in real time PCR techniques means that induced sputum PCR could provide same day results that are highly sensitive and specific, allowing for the early application of effective treatment and potentially faster recovery rates.

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