

A comparative study of methods for the diagnosis of respiratory virus infections in childhood

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In the last 20 years much progress has been made in the development of techniques for the isolation of a wide range of viruses from patients with respiratory disease (Rowe *et al.* 1953; Chanock, 1956; Chanock & Finberg, 1957; Beem *et al.* 1960; Tyrrell *et al.* 1960; Tyrrell & Bynoe, 1965). By 1965 techniques had become sufficiently advanced for a viral cause to be established in 62% of children with bronchiolitis and 42% with pneumonia (Elderkin, Gardner, Turk & White, 1965). The routine method for the investigation of children with respiratory disease has been the collection of material from the upper respiratory tract by cough and nasal (CN) swabs (Andrew & Gardner, 1963; Holzel *et al.* 1963).

In 1939, Auger devised a method for aspirating secretion from the naso-pharynx using a catheter attached to a syringe. He considered that this was an improved method for the isolation of pneumococci from children with pneumonia, although he made no direct comparison with other isolation procedures. Similar methods for obtaining secretions were later used by virologists for the isolation of parainfluenza viruses from children with croup (Morgan *et al.* 1956; Beale, McLeod, Stackiw & Rhodes, 1958; McLean *et al.* 1961); secretions are abundant and easily obtainable in this condition.

Recently, a number of workers have used nasopharyngeal secretions for rapid diagnosis. Doane *et al.* (1967) examined preparations of nasopharyngeal secretions from patients with tracheitis by electron microscopy and observed large numbers of parainfluenza virions. McQuillin & Gardner (1968) and Gardner & McQuillin (1968) examined cellular deposits from nasopharyngeal secretions by an indirect immunofluorescent technique for the rapid diagnosis of respiratory syncytial virus (RSV) infections in infants, and found many infected cells present. The large numbers of parainfluenza viruses visible by electron microscopy in nasopharyngeal secretion and of exfoliated cells exhibiting specific fluorescence for RSV suggest that this material is heavily infected. Therefore, nasopharyngeal secretions should prove to be an equal or more fruitful source of virus than swabs from the upper respiratory tract. Such aspirates may reflect more closely the pathogens present in the lower respiratory tract in bronchitis, bronchiolitis and pneumonia.

In this laboratory, nasopharyngeal (NP) secretions were taken from children mainly of 1 year of age or less who were suffering from acute lower respiratory disease. This material, examined by the fluorescent antibody technique to demon-

strate the presence of RSV, was also inoculated on tissue culture to isolate RSV or other viruses which might be associated with the illness. CN swabs, collected together in a bottle of Hanks's medium, were taken from each patient and cultured in the same manner as the NP secretions. The aim of this work was to ascertain whether NP secretions were a better source of virus than CN swabs and whether an immunofluorescent antibody technique, applied directly to the cells of the nasopharynx, compared favourably with RSV isolations in tissue culture; it was also an opportunity to assess the place of NP secretions in diagnostic virology.

METHODS AND MATERIALS

Collection of specimens

CN swabs were collected together in the same 5 ml. bottle of Hanks's medium, containing 0.2% bovine albumin, penicillin 500 units/ml., streptomycin 250 $\mu\text{g.}/\text{ml.}$ and mycostatin 50 $\mu\text{g.}/\text{ml.}$ CN swabs were always taken before the nasopharyngeal aspirates, so that the results from CN swabs would be comparable with those obtained in previous years.

NP secretions were obtained in the following way: A polythene feeding tube (size 8) was attached to a sterile plastic mucus extractor, and this in turn was connected to a suction pump, which provided a maximum negative pressure of 26 lb./in.² The feeding tube was passed through the nose into the region of the nasopharynx and when the suction was applied, the aspirate was trapped in the mucus extractor. This procedure was repeated through the other nostril. The suction pump was operating both when the tube was placed into the nasopharynx, and when it was removed from there. The mucus extractor containing the NP secretion, and the CN swab specimen in transport medium, were both carried from the wards to the laboratory in a tray of melting ice.

Preparation of specimens

The NP secretion was centrifuged in the mucus extractor at 1000 rev./min. for 10 min. at 4° C., to deposit the cells. The top of the extractor was removed with a hot wire loop, and the supernatant was separated for culture. The cellular deposit, after washing, was transferred to slides for examination by the immunofluorescent technique as described by McQuillin & Gardner (1968). A suspension of supernatant material, between 5 and 10%, was made in the Hanks's transport medium previously described.

Isolation and identification of viruses

The suspension of NP secretion and Hanks's medium containing the CN swabs were each inoculated in 0.2 ml. volumes into tissue culture tubes; the cell lines used were Bristol HeLa, HEp 2, rhesus monkey kidney and a human diploid cell line (W.I. 38). Technical procedures have been described elsewhere (Gardner & McQuillin, 1968). The CN swab specimens and suspensions of NP secretions were always inoculated on the same cell lines at the same time. Moreover, to ensure

consistency of results, each CN specimen and NP secretion was inoculated on the same batch of tubes of each particular cell line. All tubes were examined daily for cytopathic effect; W.I. 38 were kept for 10 days, monkey kidney 21 days and HeLa and HEP 2 cells were kept a total of 28 days. Should passage of the HeLa and HEP 2 cultures be required near the 28th day, then the passage tubes were kept a further 10 days.

Respiratory syncytial virus. When a giant cell degeneration compatible with that caused by RSV was observed on any of the cell lines, the agent was identified by both a neutralization test and the immunofluorescent technique. At a later stage in the investigation, when the specificity of the fluorescent antibody technique had been established, the neutralization test was discontinued.

Parainfluenza viruses. Parainfluenza viruses were isolated on rhesus monkey kidney cells. Haemadsorption tests were carried out on all monkey kidney tubes at 10 and 21 days after inoculation. Parainfluenza viruses were identified by the haemadsorption neutralization test, using parainfluenza types I, II and III antisera.

Influenza viruses. Influenza viruses were also isolated on rhesus monkey kidney cells. A complement-fixation test using influenza A, B, C and mumps antisera was performed on tissue culture fluid of haemadsorption agents which were not identified as parainfluenza viruses.

Adenoviruses. When the cytopathic effect characteristic of adenovirus degeneration was observed in tissue culture tubes, a complement-fixation test was carried out on the antigen present, using a standard positive adenovirus antiserum. The virus was then typed by a neutralization test.

Herpesvirus hominis. When a round cell degeneration occurred in tissue culture suggestive of herpesvirus hominis, the virus was identified by both a neutralization test and fluorescent antibody technique.

Rhinoviruses. Agents producing islands of round cell degeneration on W.I. 38 or monkey kidney cells were tested for their acid stability. Acid-labile viruses were then classified as M- or H-strain rhinoviruses.

Picornaviruses excluding rhinoviruses. Agents causing cytopathic changes on tissue culture suggestive of enterovirus infection were identified by a neutralization test.

Fluorescent antibody methods for the identification of viruses

The fluorescent antibody technique used in this study to identify RSV in exfoliated cells of the nasopharynx and to confirm strains of RSV and herpesvirus hominis, have been described elsewhere (McQuillin & Gardner, 1968; Gardner & McQuillin, 1968; Gardner, McQuillin, Black & Richardson, 1968).

Study group A

The study groups

NP secretions and CN swabs were collected from 111 children who were admitted to hospital in Newcastle upon Tyne with acute lower respiratory infections during 1968 (Table 1). One hundred and six children included in this series were less than

1 year of age, the oldest of the remainder being 2. NP secretions were more easily obtained from children in this age group, and this was the only selective process used in the study.

Study group B

The results for the whole of 1967 and 1968 were compared at two ages, under 1 year and over 1 year. In 1967, 99 children less than 1 year and 82 children over 1 were investigated; in 1968, the numbers were 161 and 119 respectively. Children in 1967 had only CN swabs taken but in 1968 two-thirds of the children under 1 year had NP secretions taken as well. This afforded another means of comparing the two types of specimens.

Table 1. *The clinical categories and viruses isolated from nasopharyngeal secretions and cough/nasal swabs of 111 children—Study group A*

	Clinical category				Total
	Pneu- monia	Bronchi- olitis	Bron- chitis	Croup	
Children examined	18	78	12	3	111
Children infected with virus	15 (83)	68 (87)	6 (50)	1	90 (81)
Viruses isolated					
Adenovirus	2	1	—	—	3
Parainfluenza	2*	4	1	—	7
Influenza B	—	1	—	—	1
Rhinovirus	2	—	—	—	2
Echovirus	—	2	—	—	2
RSV	9 (50)	60 (77)†	5 (42)	1	75 (68)
RSV diagnosed by fluorescent antibody method	8	58	4	1	71

Figures in parentheses are percentages of the total children in each group.

* Adenovirus type 1, herpesvirus hominis and an H-strain rhinovirus were also isolated from one of these patients.

† A poliovirus was also isolated from two of these patients, and a Coxsackievirus A9 from a third.

RESULTS

The viruses associated with acute lower respiratory disease in 111 children from whom nasopharyngeal secretions and cough/nasal swabs were taken—Study group A

Table 1 summarizes the viruses isolated from children with acute lower respiratory disease, and the clinical categories with which they were associated. When more than one virus was isolated from a child, the one considered to be the most likely aetiological agent was included in the table and others were recorded in the footnotes. A child with pneumonia was simultaneously infected with four viruses; this was her third admission to hospital with acute lower respiratory disease, her previous admissions having been attributed to RSV and parainfluenza virus

type III. This unusual sequence of events was probably related to her hypogammaglobulinaemia. Viruses were associated with 81 % of all lower respiratory infections; the individual figures were 87 % for bronchiolitis and 83 % for pneumonia.

If CN swabs alone had been used a viral cause would have been established in only 68 % of patients (76 out of 111) as compared with 79 % (88 out of 111) when NP secretions alone from the same patients were examined. Croup was associated with older children but few NP secretions were taken from patients in this clinical category.

Table 2. Comparison of methods for the diagnosis of virus infections—
Study group A

Viruses	Viruses isolated from nasopharyngeal secretions	Viruses isolated from cough/nasal swabs	Positive secretions by fluorescent antibody technique	Total no. of viruses isolated from patients
RSV	74	64	71	75
Parainfluenza type I	1	1	—	1
II	1	1	—	1
III	5	5	—	5
Adenovirus type 1	1	1	—	1*
2	—	1	—	1
4	1	—	—	1
5	1	—	—	1
Rhinovirus M-strain	2	2	—	2
H-strain	1	—	—	1*
Influenza B virus	1	—	—	1
Herpesvirus hominis	—	1	—	1*
Echovirus type 12	1	1	—	1
19	1	1	—	1
Coxsackie A virus type 9	1	—	—	1*
Poliovirus	2	2	—	2*
Total no. of viruses	93	80	71	96

* These viruses occurred in multiple infections.

Respiratory syncytial virus

RSV was the virus most commonly associated with all forms of acute lower respiratory disease in this age group, infecting 67.5 % of all these patients, including 77 % of those with bronchiolitis and 50 % of those with pneumonia. These results were based solely on the isolation of viruses; serological results were not included. Seventy-one of the 75 RSV infections (95 %) were diagnosed on the patient's day of admission by the fluorescent antibody technique. All positive fluorescent antibody results were later confirmed by isolation of the virus.

Table 2 shows that of the 75 isolations of RSV, 74 were made from NP secretions and 64 from CN swabs. There is a significant difference in the isolation rates for the two types of specimens ($\chi^2 = 7.003$, $P < 0.01$).

RSV was isolated on Bristol HeLa, HEp 2, rhesus monkey kidney and occasionally W.I. 38 cells. Table 3 shows the number of days necessary to isolate RSV on

Table 3. *The time taken for the isolation of RSV on Bristol HeLa and HEp 2 cells—Study group A*

Time of isolation (days)	Nos. of isolations on			
	Bristol HeLa cells inoculated with		HEp 2 cells inoculated with	
	NP secretion	CN swabs	NP secretion	CN swabs
0-5	1	0	1	0
6-10	28	10	26	8
11-15	22	22	24	19
16-20	11	16	11	16
21-25	3	10	4	4
26-30	2	4	3	2
31-35	0	0	1	2
Total isolations	67	62	70	51
Percentage positive by tenth day	43	16	38.5	15.5

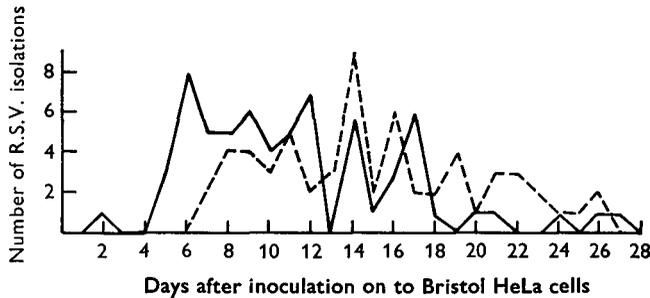


Fig. 1. The isolation of RSV on Bristol HeLa cells. Comparison between the number of days taken to isolate RSV on Bristol HeLa cells from nasopharyngeal secretion and cough/nasal swabs. —, Represents tissue cultures inoculated with nasopharyngeal secretion; - - -, represents tissue cultures inoculated with cough/nasal swabs.

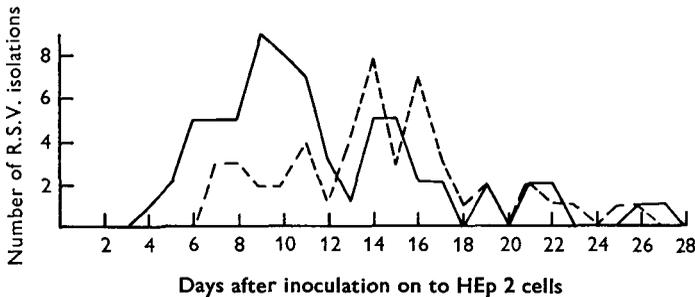


Fig. 2. The isolation of RSV on HEp 2 cells. Comparison between the number of days taken to isolate RSV on HEp 2 cells from nasopharyngeal secretions and cough/nasal swabs. —, Represents tissue cultures inoculated with nasopharyngeal secretion; - - -, represents tissue cultures inoculated with cough/nasal swabs.

HEp 2 and Bristol HeLa cells when inoculated with either NP secretion or CN swab. Forty-three per cent of isolations on Bristol HeLa cells occurred within 10 days of inoculation with NP secretion, in contrast to 16% from CN swabs. Similar figures were obtained on HEp 2 cells. Virus was isolated from two NP secretions on the second and fourth day after inoculation. Figures 1 and 2 show these differences in graphic form.

Parainfluenza viruses

A total of seven parainfluenza viruses were recovered from the 111 children examined (Tables 1 and 2). All the parainfluenza viruses were isolated on rhesus monkey kidney cells from both CN swabs and NP secretion. Two parainfluenza viruses type III were recovered from NP secretions on HEp 2 and Bristol HeLa cells, but not from the CN swabs. The child infected with a parainfluenza virus type 1 was also shown to be infected with an adenovirus type 1, an H-strain rhinovirus and herpesvirus hominis.

Adenoviruses

Adenoviruses of types 1, 2, 4 and 5 were recovered from four patients. Types 1 and 2 were recovered from CN swabs and types 1, 4 and 5 were isolated from NP secretions (Table 2).

Influenza B virus

An influenza B virus was isolated from a patient with bronchiolitis. The virus was grown on rhesus monkey kidney cells inoculated with the material from the NP secretion; the virus was not isolated from the CN swabs taken at the same time (Table 2).

Picornaviruses

Rhinoviruses. The two M-strain rhinoviruses were isolated on rhesus monkey kidney cells and W.I. 38 cells within 5 days of inoculation from both types of specimens.

The H-strain rhinovirus, isolated from the child with the multiple virus infection, was only recovered from the NP secretion on W.I. 38 cells. Herpesvirus hominis was isolated from the parallel CN swab on W.I. 38 cells.

Enteroviruses. Two echoviruses were isolated from children with bronchiolitis from both CN swabs and NP secretion. Two further children with bronchiolitis from whom RSV was isolated were also infected with two types of poliovirus, almost certainly vaccine strains. Coxsackievirus A 9 was recovered from the NP secretions of another child with bronchiolitis; this virus was not isolated from the parallel CN swab. RSV was also isolated from this child.

Comparison of virus isolations in 1967 with those in 1968—Study group B

From January to December 1967, the majority of children under the age of 1 year admitted to hospital with acute lower respiratory infection were investigated only by CN swabs, but from January to December 1968 the majority also had

NP secretions taken. Table 4 shows all the viruses which were encountered in acute lower respiratory tract infections during these 2 years and also compares the percentage virus isolations using two different methods of collecting specimens. In 1967, only 53 (54%) viruses were associated with 99 illnesses when CN swabs were used but in 1968, 123 (76%) were found in 161 illnesses when NP secretions were first introduced for virus isolation. There is a significant statistical difference in the virus isolation rate for the 2 years ($\chi^2 = 14.44$, $P < 0.0005$). RSV shows this increased isolation rate well, but though the number of other viruses isolated is small, there is a significant increase in isolation rate of those which were considered pathogenic, viz. influenza, parainfluenza and rhinoviruses.

Table 4. *A comparison of virus isolations from children under the age of 1 year with lower respiratory disease, for the years 1967 and 1968—Study group B*

Clinical category	No. in each category	Viruses						Total	%
		RSV	Para-influenza	Adeno-virus	Rhino-virus	Influenza	Entero-virus		
Pneumonia	45*	13	1	3	0	0	1	18	40
	28*	13	1	0	2	2	0	18	64
Bronchiolitis	37	24	0	0	0	0	2	26	70
	91	76	4	1	0	1	1	83	91
Bronchitis	14	6	1	1	0	0	0	8	54
	32	10	1	1	1	0	0	13	41
Croup	3	1	0	0	0	0	0	1	33
	10	4	3	0	1	0	1	9	90
Total	1967 99	44	2	4	0	0	3	53	54
	1968 161	103	9	2	4	3	2	123	76

* For each clinical category the upper line gives the figures for 1967, and the lower those for 1968.

Few NP secretions could be taken from children over the age of 1 year, so that the isolation rates for 1967 and 1968 represented the efficiency of CN swabs at this age level. In 1967, 19 of 82 children older than 1 year showed evidence of a virus aetiology (23%) and the figure for 1968 was 121 children investigated, with 33 isolations (27%). There is no significant difference in the virus isolation rates for the 2 years; this contrasts sharply with the improvement in isolation rates in children under the age of 1 year, when the taking of NP secretions was introduced as a method for virus isolation.

DISCUSSION

Over the past year, 111 secretions were obtained from children mainly under the age of one year, admitted to hospital with acute lower respiratory disease (Study group A). Secretions were selected as it had been shown previously that this type of specimen was more suitable than throat swabs for direct examination by the fluorescent antibody technique for the rapid diagnosis of RSV infection. In this

series all patients had both NP secretions and CN swabs taken; these were used to isolate any viruses which may have been present in the respiratory tract of the patients and to confirm that those secretions which gave positive fluorescence with RSV antiserum, were due to RSV infection. This afforded a means of comparing the suitability of NP secretions with CN swabs for the isolation of viruses.

The collection of NP secretions from children depends upon two factors, the patient's co-operation and the amount of secretion present in the nasopharynx. These difficulties are not normally encountered in the taking of CN swabs. In the age group studied, aspiration of secretions did not present many problems and secretions were abundant in the majority of patients with respiratory disease.

The investigation demonstrated an increase in total virus isolations, and RSV in particular, from NP secretions as compared with CN swabs (Table 2). The introduction of NP secretions in addition to CN swabs for the isolation of viruses resulted in a considerable increase in our knowledge of the viral causes of both bronchiolitis and pneumonia as compared with our experience and the experience of others over previous years (Chanock *et al.* 1961; Holzel *et al.* 1963, 1965; Elderkin *et al.* 1965; Holdaway, Romer & Gardner, 1967). Evidence for this was gained from Study group B by comparing the total virus isolations for the year 1967 with those for 1968 (Table 4). There was a significant increase in the virus isolations from children under 1 year of age in 1968 in comparison with 1967, a year when only CN swabs were used. There was no significant increase in 1968 in virus isolations from children over 1 year of age, from whom only CN swabs were taken.

When NP secretions and CN swabs from children with RSV infections were inoculated on HEp 2 and Bristol HeLa cells, the virus was recovered much more rapidly from the tissue cultures inoculated with NP secretions (Figs. 1 and 2, Table 3). This was an additional reason for advocating the use of NP secretions whenever possible. More than one cell line was always inoculated for the isolation of RSV, since the presence of inhibitory substances and the variation in the sensitivity of cells are both factors influencing its growth in tissue culture (Jordan, 1962). The most efficient method for the isolation of RSV was by the inoculation of NP secretions on HEp 2 cells, when 70 viruses out of a possible 75 were isolated. However, the number of positive results obtained by the direct examination of NP secretions by the fluorescent antibody technique was marginally greater than this and 95% of those with RSV infections were diagnosed on the day of their admission to hospital. In the future, there would appear to be justification for discontinuing the culture of RSV from secretions which are positive by the fluorescent antibody technique.

There are limitations in the application of the fluorescent antibody technique to NP secretions. The occasional false negative result or results showing only scanty positive cells may be due to the stage in the disease when the specimen was examined, difficulty in obtaining secretions, or the amount of mucus present, preventing effective staining, but continued culture of the occasional doubtful specimen should not present a problem.

Twenty-one viruses other than RSV were isolated from patients in this investiga-

tion. This small number makes similar comparisons impracticable. Beale *et al.* (1958) found that NP secretions were suitable for the isolation of parainfluenza viruses, and McLean *et al.* (1961), also using this material, recovered parainfluenza viruses from 52% of children with acute laryngotracheobronchitis. None of these workers, however, compared NP secretions with CN swabs for suitability as material for the isolation of parainfluenza viruses. In this series, all the parainfluenza virus isolations were made from both kinds of specimens on monkey kidney cells. No comparison could be made between the numbers of days required for the isolation of these viruses from the two types of specimens, since the haemadsorption test was only carried out on the tenth and twenty-first day after inoculation. However, it was found that two parainfluenza type III viruses grew on Britsol HeLa and HEp 2 cells, as well as on rhesus monkey kidney cells when the specimen used was the NP secretion; the virus did not grow on HeLa or HEp 2 cells inoculated with the parallel CN swabs. This suggested that those NP secretions contained more infective virus than the CN swabs. The large numbers of parainfluenza viruses found in NP secretions by Doane *et al.* (1967), suggest that it may be possible to apply a fluorescent antibody technique for the examination of secretions for the rapid diagnosis of parainfluenza virus infections.

Of the four adenoviruses isolated, three were non-epidemic types (types 1, 2 and 5) which are of unproven pathogenic significance (Gardner, 1968). Table 2 shows that types 1, 4 and 5 were isolated from NP secretions and types 1 and 2 from CN swabs. This is too small a difference on which to comment, but it is noteworthy that adenovirus type 4, the only epidemic strain isolated, came from a NP secretion.

The single isolation of influenza B virus was from a child with severe bronchiolitis; the virus was recovered from NP secretion, but not from the CN swab.

Rhinoviruses have been known for sometime to infect the upper respiratory tract (Tyrrell *et al.* 1960). Though Portnoy, Eckert & Salvatore (1965) suggested rhinoviruses were of no aetiological significance in lower respiratory disease of childhood, Hilleman, Reilly, Stokes & Hamparian (1963) found that 'coryza viruses' (rhinoviruses) were associated with lower respiratory tract infection in a third of a small number of children investigated, and Stott *et al.* (1967) found rhinoviruses in 7% of children with acute lower respiratory infections. In this series, three rhinoviruses were isolated from NP secretions, but only two from CN swabs (Table 2). All these three children were suffering from pneumonia, though in one child with a multiple virus infection the rhinovirus was unlikely to be the aetiological agent. This suggests that in this age group, rhinoviruses are not an important cause of lower respiratory infection but if the viruses in the nasopharynx reflect the viruses present in the lung, then rhinoviruses may be the pathogens involved in sporadic cases.

The single isolation of herpesvirus hominis was from the CN swab but not from the NP secretion.

The significance of enteroviruses is difficult to judge; they often occurred as dual infections with RSV and could be isolated easily from both types of specimen.

NP secretions occasionally had toxic effects on tissue culture, which resulted in

partial or total destruction of the cell sheet. It was found that if these tubes were passaged, particularly within the first few days after inoculation, then the isolation of RSV was considerably delayed and in one instance when the specimen was passaged twice within the first 5 days, isolation failed completely. CN swabs rarely produced toxic effects in tissue cultures; bacterial contamination only occasionally occurred with either specimen. The direct examination of NP secretions by the fluorescent antibody technique could be an additional safeguard for obtaining a positive result, should toxicity or contamination prevent the effective routine culture of specimens.

Our results have shown that NP secretions are a better source of virus and, in addition, more suitable specimens for rapid diagnostic techniques than CN swabs (McQuillin & Gardner, 1968). When all viruses are considered together, the results obtained in this investigation suggest that the examination of NP secretions reflects more accurately the aetiology of acute lower respiratory disease.

SUMMARY

Nasopharyngeal secretions and cough/nasal swabs were taken from 111 children admitted to hospital in Newcastle upon Tyne with acute lower respiratory disease. A comparison was made between nasopharyngeal secretions and cough/nasal swabs as material for isolation of viruses in tissue culture. These results were, in turn, compared with those obtained by applying a fluorescent antibody technique to the exfoliated cells in the nasopharyngeal secretions for the rapid diagnosis of respiratory syncytial virus infection.

More viruses were isolated in tissue culture from nasopharyngeal secretion than from cough/nasal swabs. Further evidence for the superiority of nasopharyngeal secretions was obtained by comparing the virus isolations in the laboratory in 1967 with those in 1968. Respiratory syncytial virus was not only isolated more often but more quickly in tissue culture inoculated with nasopharyngeal secretions.

The fluorescent antibody technique not only provided a diagnosis on the patient's day of admission in 95 % of those infected with respiratory syncytial virus but also proved to be as sensitive as the culture of nasopharyngeal secretions and considerably more sensitive than the culture of cough/nasal swabs for the diagnosis of respiratory syncytial virus infection.

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