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RAPID DETECTION OF RESPIRATORY SYNCYTIAL VIRUS IN CLINICAL SPECIMENS

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Summary. — Results of the direct immunofluorescence (IF) test in 152 clinical specimens (throat swabs) were compared with those of respiratory syncytial virus (RSV) isolation. The prevalence of RSV infections in the upper and lower respiratory tract was high especially in infants until 12 months of age. The average RSV isolation rate was 18.42%, whereas the virus antigen detection was positive in 19.74% of cases. The agreement between virus isolation and direct IF was 92.1%, the sensitivity of IF being 82.14% and its specificity 94.35%.

Key words: respiratory syncytial virus, immunofluorescence, diagnosis of RSV infections

Introduction

While in adults RSV causes mild respiratory tract infections (mainly reinfections), in children, and especially infants, it tends to cause acute bronchiolitis (25—50 % of cases), interstitial pneumonia (12.5—25 % of cases) as well as mild upper respiratory tract diseases in 13 % of infected children, respectively (Jackson and Muldoon, 1975). There are few reports (Brandt *et al.*, 1973, Chanock *et al.*, 1951, Jacobs *et al.*, 1971) of asymptomatic respiratory tract affection in children.

The optimal diagnostic procedures for RSV are virus demonstration by isolation techniques and the methods using labelled hyperimmune sera. The detection of RSV by labelled antibodies complements the isolation procedure; it is quick and demonstrates the presence of antigen even when virus infectivity had been lost (Gardner and McQuillin, 1974). Both, immunofluorescence (IF) and ELISA are the methods of choice, the latter being less sensitive in antigen detection than the former (Hornsleth *et al.*, 1982).

An endeavour was made to improve the quality of rabbit RSV antiserum for direct IF by reducing the nonspecific staining of epithelial cells in the

smears and by preparing a conjugate from hyperimmune serum of high antibody titre. The prevalence of RSV infection among hospitalized cases was investigated comparing the results of RSV isolation technique and direct IF in clinical specimens.

Materials and Methods

RSV cultivation. RSV strain Long was used. Virus was grown in continuous cell lines (Pons *et al.*, 1983): HEp-2, HeLa, GMK, Vero, human skin fibroblasts, FL, BEK, rabbit kidney (RK-13) and in primary chicken fibroblast cell culture. All cells were cultured in Eagle's MEM, containing 10% calf serum and antibiotics (penicillin and streptomycin) and maintained in the same nutrient medium supplemented with 2% calf serum and 0.3% glutamine.

Immunization of rabbits. For immunization, RSV was cultivated in HEp-2 and Vero cells The inoculum (log $TCID_{50}$ of 2.86 and 2.9, respectively), was prepared according to Gardner and McQuillin (1974).

Immunization schedule. RSV replicated in HEp-2 (trial A) or Vero (trial B) cells was mixed with equal amount of complete Freund's adjuvant and inoculated intramuscularly (0.9 ml into gluteal area), and subcutaneously (0.1 ml into hind foot pads). Booster dose was administered 6 weeks later containing fresh RSV in incomplete Freund's adjuvant, the second booster was repeated after 4 weeks, i.e. blood was drawn after 11 weeks.

Absorption of hyperimmune serum. Serum absorption was done to HEp-2 and HeLa cells in trial A and to HEp-2 and Vero cells in trial B according to the Gardner and McQuillin (1974).

Labelling of hyperimmune serum. Hyperimmune serum obtained in trial B was used for labelling. The serum was precipitated with ammonium sulphate (Nairn, 1976). The gammaglobulin fraction was conjugated with FITC (fluorescein isothiocyanate isomer 1-BDH, GB) in the ratio of 12 mg FITC/g protein. Unbound dye was removed by filtration through a Sephadex G-25 column. The molar FITC/protein ratio of the conjugate was 1.8. Control (preinoculation) rabbit serum was labelled in the same way.

Control tests. The specificity of hyperimmune serum was tested on cell cultures infected with the following viruses: adenovirus types 2 and 7, herpes simplex virus type 1, parainfluenza virus type 2, and poliovirus type 2. The presence of antibodies against influenza virus types A and B was tested by counter current immunoelectrophoresis using allantoic liquid from infected fertile hen's eggs as antigen.

Clinical specimens. The study was conducted by taking 217 throat swabs. The study period coincided with the season of RSV-infection (from September 1, 1982 to May 1, 1984). Specimens were taken from patients hospitalized in Zagreb. The collected swabs were placed into a test tube with transportation medium (Hanks' saline supplemented with 0.5% chicken serum and antibiotics). The swab was kept at 4 °C for several hr (3—4) until delivery to the laboratory. Transportation medium from clinical specimens was centrifuged at 3000 rev/min for 20 min.

Virus isolation. The supernatant obtained by centrifugation of clinical specimens was inoculated into tubes containing HeLa and GMK cells. Nutrient medium consisting of Eagle's MEM in Hanks' saline, 10% calf serum, 0.001% arginine (for GMK cells), 0.03% glutamine (for HeLa cells) and antibiotics (penicillin and streptomycin) was used for cell cultivation. For maintenance of cell culture the same MEM with 2% chicken serum was used. Inoculated cell cultures were incubated at 37 °C until CPE was observed. Isolated RSV was demonstrated by CF using specific rabbit hyperimmune serum.

Virus antigen detection by IF. Sediments from eluted throat swabs served for demonstration of RSV antigen by direct IF (Gardner and McQuillin, 1974; Minnich nad Ray, 1980). In this test our own conjugate diluted 1 : 40 (optimum staining titre) was used.

Mounted preparations were examined in Leitz Dialux 20 fluorescent microscope, at magnification ×400.

Statistical evaluation. All clinical specimens, i.e. the 217 throat swabs, were examined by RSV isolation as well as by indirect IF in a double blind test. The IF method was evaluated against the standard virus isolation method by following parameters: 1. IF test sensitivity (Se), 2. IF test specificity (Sp), 3. agreement between the two methods (isolation and IF) (Mučič *et al.*, 1973), 4. McNamara's χ^2 test for dependent samples (Conover, 1971).

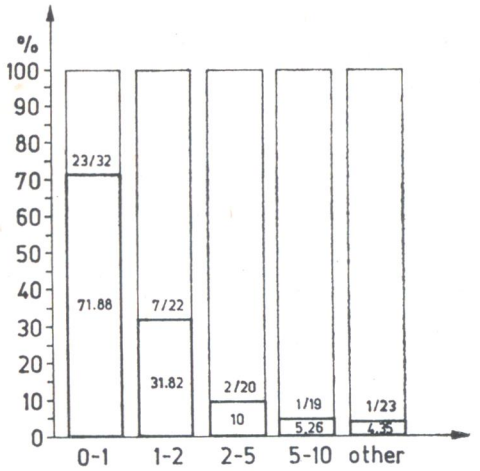

Fig. 1.
Positive RSV findings obtained by isolation and/or direct IF in clinical specimens from patients according to age. The number of clinical specimens equals the number of acute respiratory illness group specimens minus four specimens without the indication of patient age, i.e. a total of 116 specimens.
Abscissa: age (years); ordinate: per cent positivity.

Results

Production of anti-RSV hyperimmune serum

In HEp-2, HeLa, GMK and Vero cell cultures, CPE manifested itself on the second day following inoculation and progressed over the next two days to develop large syncytia. Starlike processes of syncytial cells were

Table 1. Clinical diagnosis, RSV isolation and direct IF results

Acute respiratory disease	Diagnosis	No. of throat swabs	RSV isolation		Positive direct IF	
			No.	(%)	No.	(%)
Yes	Febrile respiratory disease	58	12	(20.7)	16	(27.6)
	Influenza-like syndrome	37	5	(13.5)	5	(13.5)
	Pneumonia, bronchopneumonia	8	5	(62.5)	4	(50.0)
	Nonspecific febrile infections	7	1	(14.3)	0	0
	Bronchitis, bronchiolitis	5	3	(60.0)	2	(40.0)
	Pertussis syndrome	3	1	(33.3)	1	(33.3)
	Tonsillopharyngitis	2	0	0	1	(50.0)
No	Herpetic gingivostomatitis	9	0	0	0	0
	Fever and exanthema	5	0	0	0	0
	Meningoencephalitis	5	0	0	0	0
	Epidemic parotitis	1	0	0	0	0
	Unclassified (no diagnosis)	12	1	(8.3)	1	(8.3)
	No. of tested throat swabs	152				
	No. of RSV isolations		28	(18.4)		
	No. of positive direct IF results				30	(19.7)

Table 2. Comparison of RSV isolation and direct IF

		Direct IF	
No. of throats swabs	152	posit. 30	negat. 122
RSV isolation	posit. 28	23	5
	negat. 124	7	117
Sensitivity (Se)		82.14%	
Specificity (Sp)			94.35%
Agreement between isolation and direct IF		92.10%	
χ^2_{McN}		0.083	
df		1	
P		> 0.05	

seen in human skin fibroblasts somewhat later, i.e. on the days 5—7 post-infection (p.i.).

CPE did not develop in FL cell culture, though the virus replicated in the cells as shown by IF in FL cells. In BEK cells the CPE failed to develop even after 6 consecutive RSV passages. The virus, nevertheless, replicated in these cells as determined by IF at third virus passage.

RSV failed to grow in the continuous RK-13 line. For example, in the course of five consecutive virus passages in RK-13 cells examination by IF technique showed no positive results. No RSV replication was observed in primary chicken fibroblast culture and no virus antigen was detectable in these cells by direct IF.

Hyperimmune serum prepared according to the immunization schedule in trial A showed a CF anti-RSV titre of 1 : 256. The serum was absorbed twice to HEp-2 and HeLa cells in order to remove undesirable anti-cell antibodies. Hyperimmune sera obtained in trial B immunization schedule showed a CF-specific antibody titre ranging from 1 : 128 to 1 : 256. The sera had to be absorbed only once to HEp-2 and Vero cells.

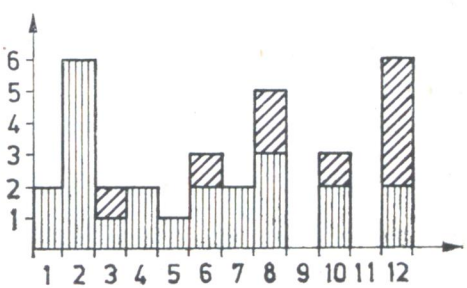

Fig. 2.
Positive RSV findings obtained by virus isolation and/or direct IF in clinical specimens from infants.
Perpendicular lines: positive specimens; dashed lines: negative specimens. The number of clinical specimens equal the number of acute respiratory illness specimens minus four specimens without indication of patient age i.e. a total of 32 specimens.
Abscissa: age in months; ordinate: no. of specimens.

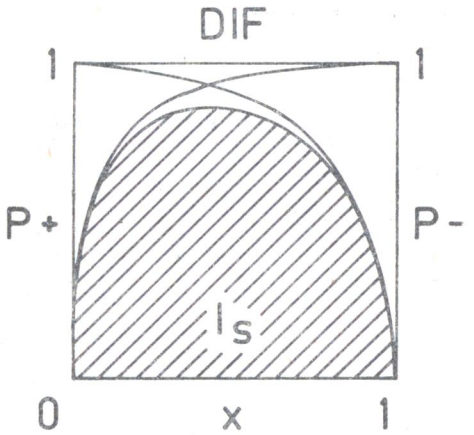

Fig. 3.
Diagnostic validity (Is) of direct IF. Integral S (Is) surface beneath the curve S corresponds to test's overall diagnostic value, (p +) probability of true positives in respect to all positives obtained in clinical specimens, (p −) probability of true negatives in respect to all negatives obtained in clinical specimens, (X) isolation rate for RSV in clinical specimens.

Demonstration of RSV in clinical specimens

All clinical specimens were examined by virus isolation in cell culture (Table 1).

The same specimens were investigated by direct IF using hyperimmune serum prepared as described above. The results were only considered positive where a minimum of two undamaged ciliated epithelial cells with intracytoplasmic inclusions were found in the spotted swab sediment. In nearly a third (30 %) of all swabs the number of ciliated cells was insufficient.

As shown in Table 2, the findings obtained by virus isolation and direct IF in 152 throat swabs were compared. The sensitivity of direct IF was 82.14 %, specificity 94.35 % and the agreement with the isolation method was 92.10 %. McNamara's χ^2 test for direct IF was 0.083, df = 1, $P > 0.05$, which indicates that there was no statistically significant difference between the results as obtained by both methods.

Frequency of RSV infections

The 152 throat swabs from hospitalized patients were coming from various clinical diagnoses (Table 1). Either by viral isolation and/or direct IF positive RSV was found in cases of various respiratory syndromes only, i.e. febrile respiratory disease, influenza-like syndrome, pneumonia, nonspecific febrile infections (NFI), bronchiolitis, pertussis syndrome and tonsillopharyngitis.

Fig. 1 shows the frequency of RpV-positive findings by age. The number of positive findings (virus isolation and/or direct IF) was the greatest in the first year of life (23/32 positive findings, i.e. 71.88 %). Fig. 2 shows the frequency of positive RSV findings in infancy. The majority of such findings was related to the second month of life. In fact, all collected clinical specimens (6 throat swabs) with relevant clinical diagnoses of RSV infection from children aged 2 month were found to be positive by both virus isolation and/or by direct IF.

Discussion

With regard to the ability to produce CPE in human (HEp-2, HeLa, skin fibroblast) and monkey (Vero) cell cultures, both can be used for growing RSV to prepare immunization antigen. Although the virus inoculum was purified by centrifugation (Gardner and McQuillin, 1974), cellular impurities remained. For this reason, antibodies to human epithelial cells may be present in nonabsorbed antiviral serum obtained from rabbits immunized with HEp-2 grown RSV; nonspecific antibodies produce undesirable immunofluorescence in clinical specimens. RSV also produces CPE in Vero cells providing an opportunity to eliminate this kind of heterologous fluorescece. Hence, anti-monkey but not anti-human cellular antibodies are produced in rabbit; nevertheless, antiviral serum with anti-monkey antibodies reacts nonspecifically with human epithelial cells though to a lesser degree than the immune serum containing anti-HEp-2 cell antibodies. Such, though milder, heterologous immunofluorescence is caused by cross-compatibility of primate cells. This type of immunofluorescence when using rabbit hyperimmune serum would be minimal if RSV could replicate in cells of avian origin (chicken fibroblasts) and even absent if RSV could grow in cell cultures of rabbit origin (RK-13).

Since nonspecific immunofluorescence in clinical specimens cannot be completely avoided, nonspecific antibodies must be removed. Therefore, it is advantageous to obtain a titre as high as possible against specific antibodies, since in the course of absorption the antibody titre decreases by about a half (Gardner and McQuillin, 1974).

Hyperimmune serum produced by our procedure resulted in a highly specific and sensitive direct IF of high diagnostic validity, i.e. diagnostic value (Is) as demonstrated on Fig. 3 (Mućić *et al.*, 1973).

The number of cells in our clinical smears for direct IF was frequently (30 % of cases) insufficient. Nasal washings (Hall and Douglas, 1975) and cough swabs (McQuillin *et al.*, 1970) were more suitable for RSV isolation, whereas nasopharyngeal secretion (Gardner and McQuillin, 1974) was most suitable for IF as it contained more cells, particularly ciliary epithelial cells susceptible to the virus.

The highest RSV infection rate was observed during the first year of life, i.e. in 23 out of 32 patients, with a peak at second month of life (Figs. 1 and 2); this was in agreement with previous reports (Jacobs *et al.*, 1971).

Our RSV positive rates (Table 1) for cases of severe lower respiratory tract infections were higher than described in literature, i.e. 60, 62.50 and 33.33 % for bronchiolitis, pneumonia and symptoms of whooping cough, respectively (Chanock *et al.*, 1961). Nevertheless, the positive rate of even mild upper respiratory tract RSV infections was rather high: catarrhal febrile disease (27.59 %), NFI (14.29 %) and influenza like symptoms (13.51 %).

Biological characteristics point to presence of a "wild" RSV strain which had circulated in Zagreb area (population of about 1 million) during the 1983/

/84 winter season. As noted earlier, virulence of RSV tended to show seasonal variation (Yurlova *et al.*, 1983).

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